Analytical Profiles of Drug Substances

Volume 15

Edited by

Klaus Florey

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PREFACE

Although the official compendia define a drug substance as to identity, purity, strength, and quality, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. Such information is scattered through the scientific literature and the files of pharmaceutical laboratories.

I perceived a need to supplement the official compendial standards of drug substances with a comprehensive review of such information, and fifteen years ago the first volume of *Analytical Profiles of Drug Substances* was published under the auspices of the Pharmaceutical Analysis and Control Section of the APhA Academy of Pharmaceutical Sciences. That we were able to publish one volume per year is a tribute to the diligence of the editors to solicit articles and even more so to the enthusiastic response of our authors, an international group associated with pharmaceutical firms, academic institutions, and compendial authorities. I would like to express my sincere gratitude to them for making this venture possible.

Over the years, we have had queries concerning our publication policy. Our goal is to cover all drug substances of medical value and, therefore, we have welcomed any articles of interest to an individual contributor. We also have endeavored to solicit profiles of the most useful and used medicines, but many in this category still need to be profiled.

Klaus Florey

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AMILORIDE HYDROCHLORIDE

David J. Mazzo

1. Introduction

1.1 Therapeutic Category (1)

Amiloride hydrochloride dihydrate, hereafter referred to as amiloride hydrochloride, is a potassium-conserving diuretic with relatively weak natriuretic and antihypertensive activity. It is not an aldosterone antagonist and, therefore, is effective in the absence of aldosterone. Amiloride hydrochloride is indicated as adjunctive treatment with thiazide diuretics or other kaliuretic-diuretic agents in congestive heart failure or hypertension to aid in the restoration of normal serum potassium levels and/or to prevent the development of hypokalemia. Amiloride hydrochloride is available for oral dosing as tablets, is usually well tolerated, and except for hyperkalemia, has had significant adverse effects reported infrequently (1).

1.2 History

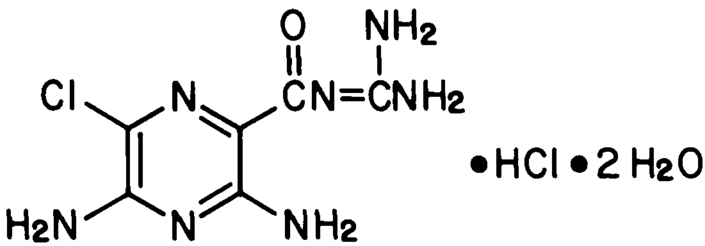
Amiloride hydrochloride, a substituted (pyrazinecarbonyl)guanidine, was first synthesized in the Merck, Sharp and Dohme Research Laboratories (2). The first non-patent literature reference to amiloride appeared in 1966 (3) and the first structure-activity relationship study was published in 1967 (4). Amiloride hydrochloride has gained steadily increasing popularity as a therapeutic drug as well as a pharmacological tool and its actions and effects have been the subject of at least three international symposia (5,6,7). A search of *Chemical Abstracts* from 1966 to 1985 produced 490 bibliographic citations for works dealing with amiloride hydrochloride.

2. Description

2.1 Chemical Name, Formula, Molecular Weight

The current accepted *Chemical Abstracts* name for amiloride hydrochloride (MK-870) is 3,5-diamino-*N*-(diaminomethylene)-6-chloropyrazinecarboxamide monohydrochloride dihydrate. The CAS registry no. is 17440-83-4.

Other names which have been used for amiloride hydrochloride include 3,5-diamino-N-(amino-iminomethyl)-6-chlorpyrazinecarboxamide monohydrochloride dihydrate, N-amidino-3,5-diamino-6-chloropyrazinecarboxamide monohydrochloride dihydrate, N-amidino-3,5-diamino-6-chloropyrazinamide monohydrochloride dihydrate, 1-(3,5-diamino-6-chloropyrazinecarboxyl)guanidine monohydrochloride dihydrate, 1-(3,5-diamino-6-chlorpyrazinoyl)guanidine monohydrochloride dihydrate, as well as the monohydrochloride dihydrated salts of guanamprazine, amipramidin and amipramizide



$C_6H_8ClN_7O \cdot HCl \cdot 2H_2O$
Molecular Weight: 302.12 g/mole

2.2 Definition

Amiloride Hydrochloride, unless specifically stated otherwise, is defined as the crystalline, monohydrochloride dihydrated salt form of the compound. Its tradename is MIDAMOR®. Amiloride, when referred to, indicates the free base.

2.3 Appearance, Color, Odor

Amiloride hydrochloride is a yellow to greenish yellow crystalline powder which is odorless or practically odorless.

3. Synthesis

Amiloride hydrochloride, essentially a substituted guanidine, has been prepared through a series of synthetic steps beginning with methyl cyanoacetate and urea (4,8-14). The synthetic route is presented in

Figure 1. The starting materials (I and II) are reacted in sodium isopropoxide and subsequently nitrosated. The product (III) is reduced to the amino compound (IV), treated with glyoxal to form a pteridine intermediate (V) and hydrolyzed in base which upon acidification gives 3-aminopyrazinoic acid (VI). This substituted pyrazinoic acid is then esterified with methanol (VII) in the presence of sulfuric acid, chlorinated (VIII) and converted to the 5-amino ester with ammonia in DMSO. The product (IX) is then reacted with guanidine to form amiloride which upon reaction with hydrochloric acid in water forms amiloride monohydrochloride dihydrate (X).

4. Physical Properties

4.1 Infrared Spectrum

The infrared spectrum of amiloride hydrochloride taken in a KBr pellet is shown in Figure 2 (15). A Digilab Model FTS-15C fourier transform infrared spectrophotometer was used to acquire the spectrum. Frequency assignments for some of the characteristic bands are listed in Table I.

Table I
Infrared Spectral Assignments for Amiloride Hydrochloride

Frequency (cm^{-1})	Assignment
3250-3500	N-H stretch (NH_2)
3150	N-H stretch (NH)
1680	C=O stretch
1640	H_2 deformation mode
1600	H_2 deformation mode
1240	N-(C_6H_6) stretch
770	C-H out-of-plane mode

The infrared spectrum of amiloride hydrochloride taken in a mineral oil mull is shown in Figure 3 (15).

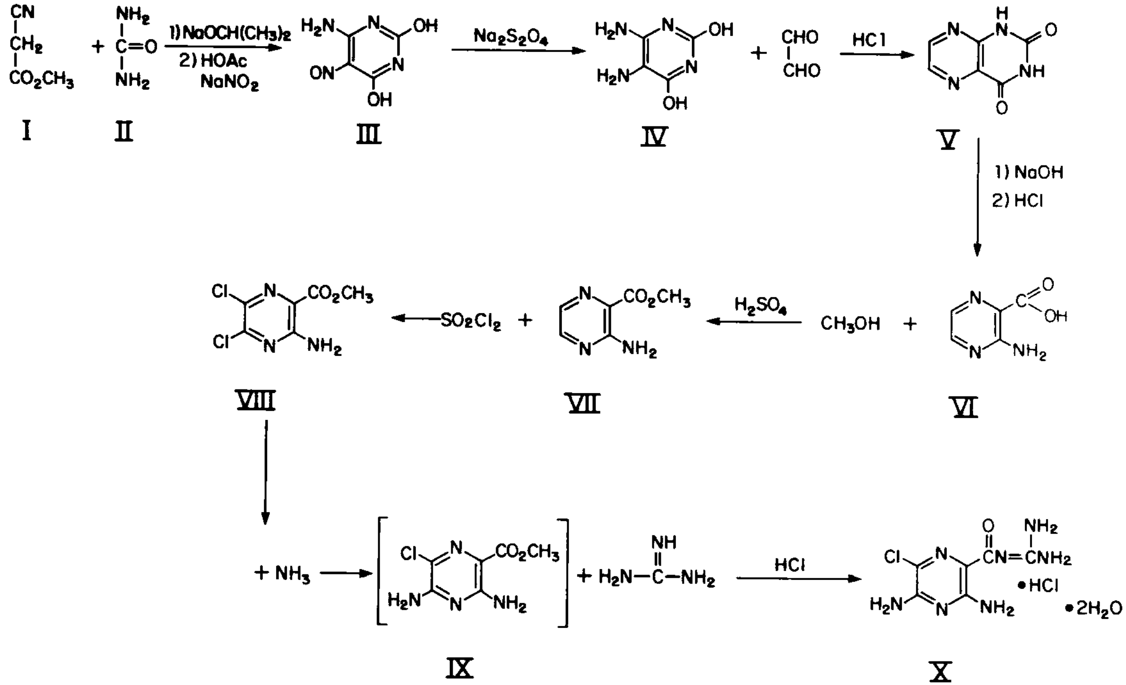


Figure 1. Synthetic route to amiloride hydrochloride

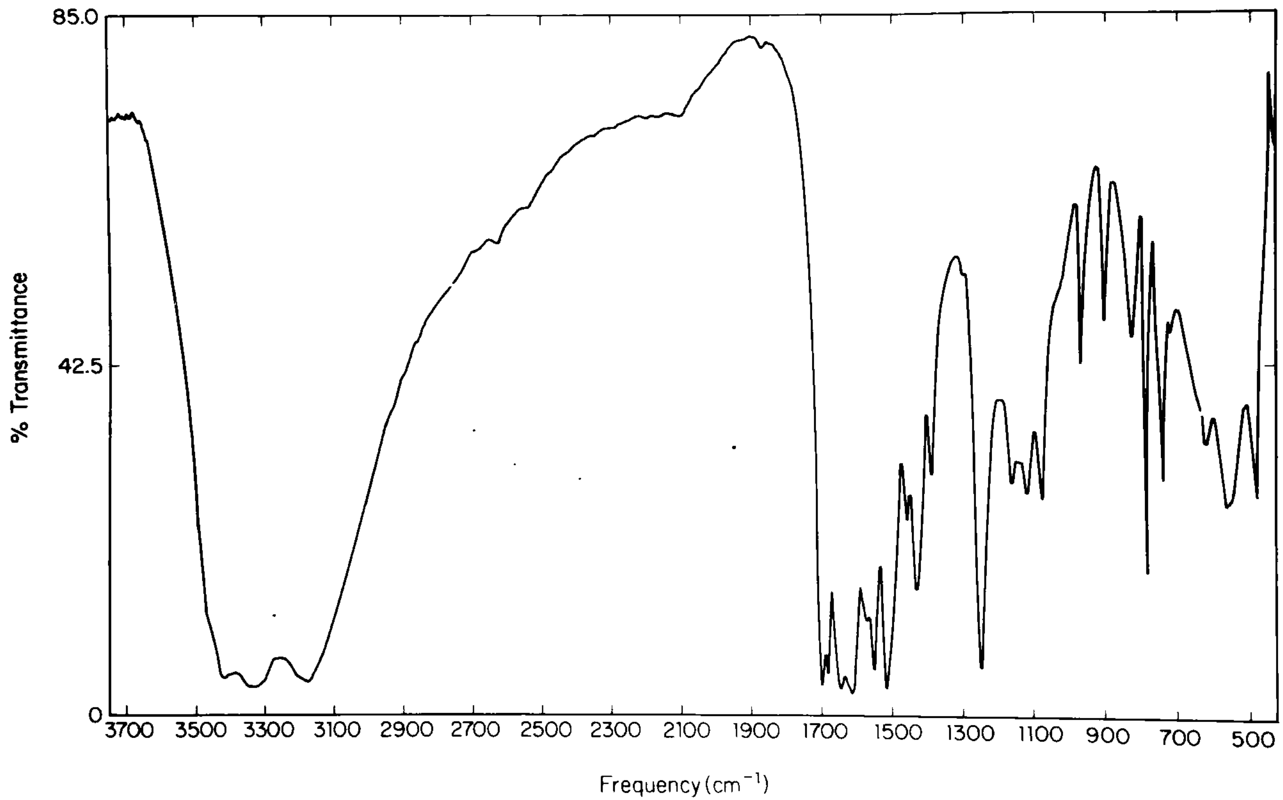


Figure 2. Infrared spectrum of amiloride hydrochloride taken in a KBr pellet.

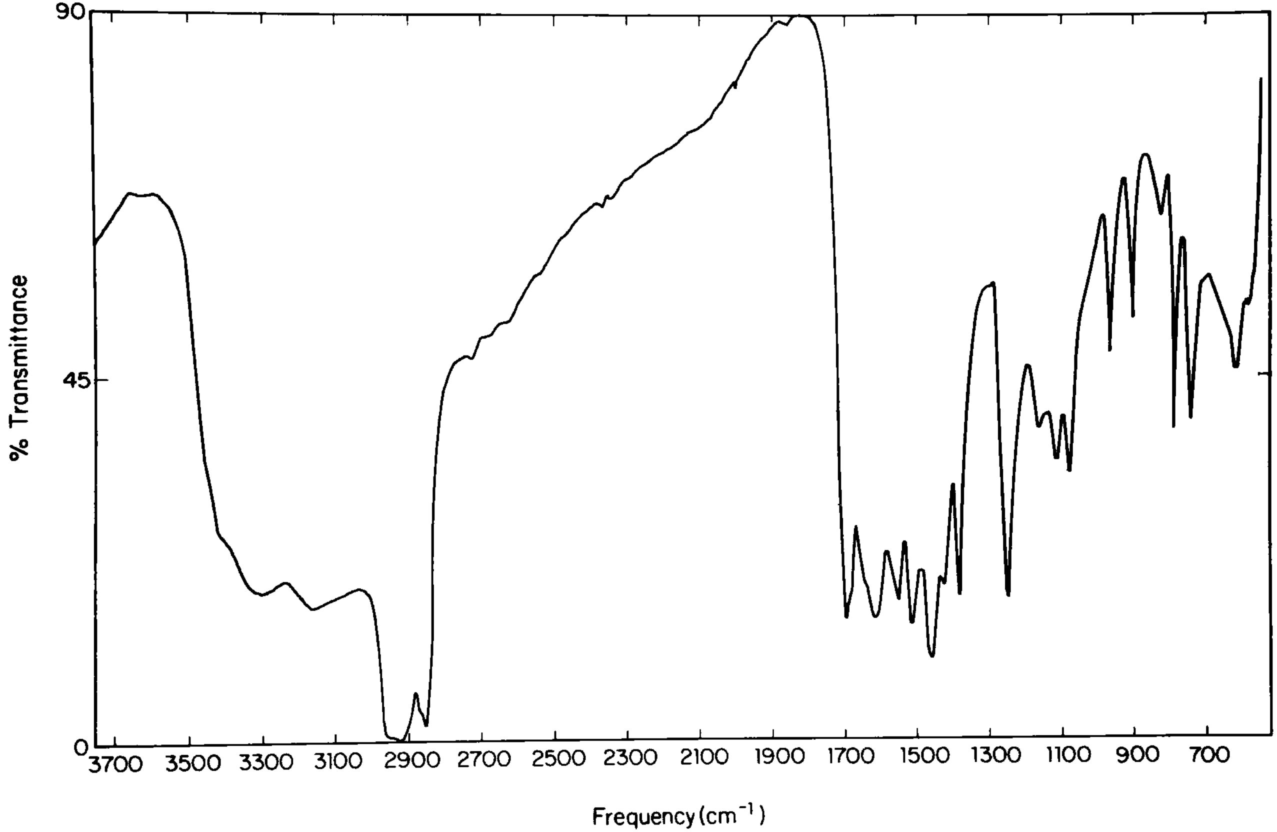


Figure 3. Infrared spectrum of amiloride hydrochloride taken in a mineral oil mull.

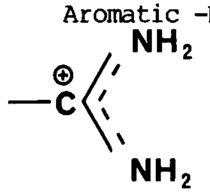
4.2 Nuclear Magnetic Resonance Spectrum (16)

4.2.1 Proton NMR Spectrum

The proton magnetic resonance spectrum of amiloride hydrochloride was obtained using a JEOL(USA) C-60HL spectrometer. The spectrum was acquired from a 1.0 M drug solution in fully deuterated dimethylsulfoxide (d_6-DMSO). The spectrum is shown in Figure 4 and the spectral assignments are listed in Table II (17).

Table II

Proton NMR Spectral Assignments for Amiloride Hydrochloride

(ppm)[a]	Integral (mm)	Relative No. Protons	Assignments
3.8	52	4.4[b]	H_2O
7.4			
	48.5	4.1	Aromatic $-NH_2$
7.7			
9.8			
	57[c]	4.9	
11.1			N—H

Notes:

(a) All protons are bound to nitrogen or oxygen (H_2O), therefore chemical shift will be strongly dependent upon sample concentration, temperature and/or solution pH.

(b) This signal includes a small contribution due to water originally present in the d_6-DMSO.

(c) The total integral resulting from these signals is reported since the appearance of the spectrum suggests intermediate kinetic exchange between the two types of sites.

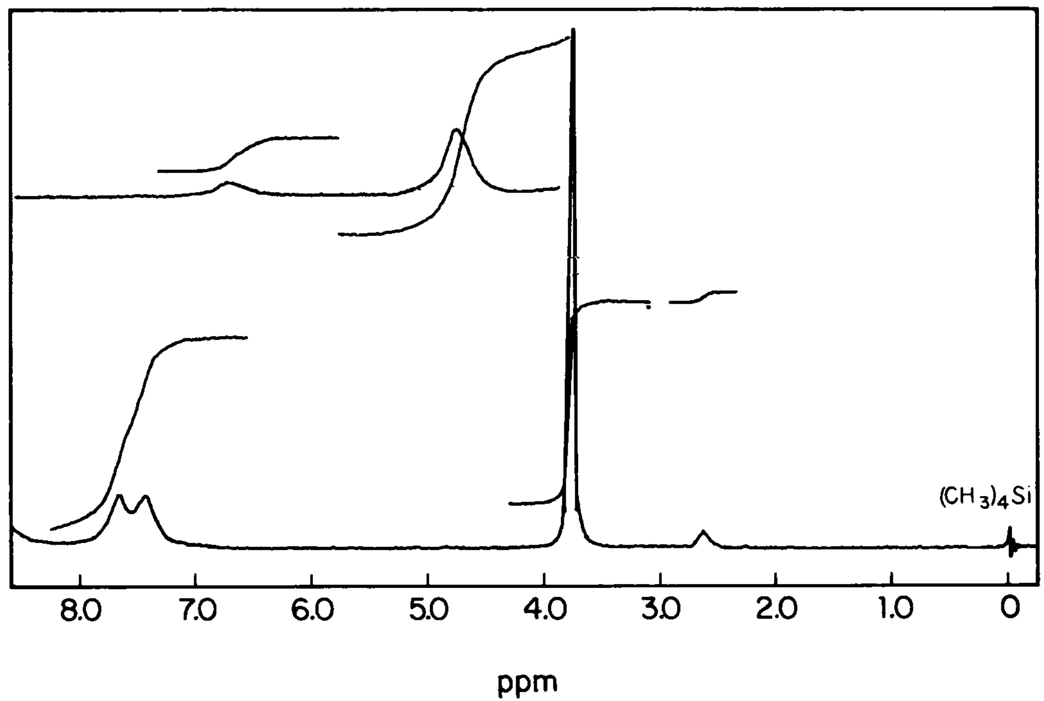


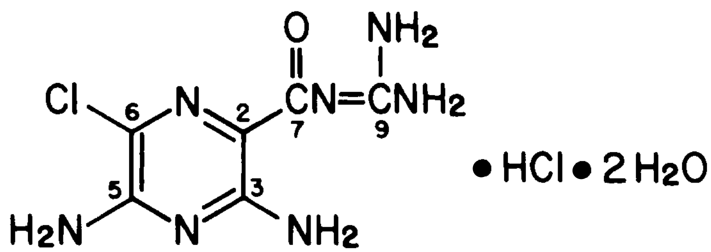
Figure 4. Proton NMR spectrum of amiloride hydrochloride.

4.2.2 C^{13} NMR Spectrum

The C^{13} NMR spectrum shown in Figure 5 was obtained on a Varian Associates CFT-20 spectrometer using a 1.0 M amiloride hydrochloride solution in d_6-DMSO (17). The C^{13} spectral assignments are listed in Table III.

Table III
C^{13} NMR Spectral Assignments for Amiloride Hydrochloride

(ppm)	Assignments
109.3	C_2
119.9	C_6
154.2	C_3
155.3	C_9
155.9	C_5
165.3	C_7



Both the proton and C^{13} NMR spectra are consistent with the amiloride hydrochloride structure.

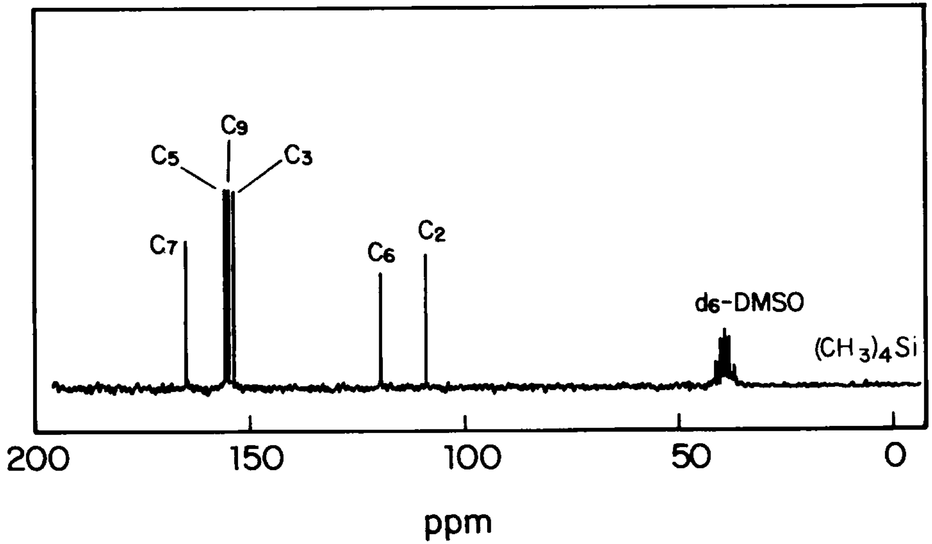


Figure 5. C^{13} NMR spectrum of amiloride hydrochloride.

4.3 Ultraviolet Spectrum

The ultraviolet absorption spectrum of amiloride hydrochloride in 0.01 N aqueous hydrochloric acid is characterized by maxima at approximately 212 mm, 285 nm and 362 nm.

The absorbance in absorbance units of a 1% drug solution in a 1 cm cell ($A_{1\,cm}^{1\%}$) is 642, 555 and 617 for the wavelengths of 212 nm, 285 nm and 362 nm, respectively. The UV spectrum shown in Figure 6 was obtained using a Cary Model 18 ultraviolet spectrophotometer (18).

4.4 Mass Spectrum

Figure 7 is a depiction of the low resolution mass spectrum of amiloride hydrochloride obtained using a LKB model 9000 mass spectrometer in the electron impact mode with an ionization energy of 70 eV and a probe temperature sufficiently high to produce sample vaporization (19). The spectrum is dominated by the molecular ions of the free base (m/e = 229, 231). Masses of m/e = 43, 187, 189, 171, 173 and 212, 214 characterize the guanidino substituent. Loss of the entire guanidino group leads to m/e = 144, 146 and m/e = 86.

The remaining less abundant ions are most likely due to losses of CH_2N from the pyrazine ring, although the loss of CO cannot be totally excluded on the basis of low resolution spectra. Figure 8 schematically presents the fragmentation of amiloride hydrochloride.

4.5 Optical Rotation

Amiloride hydrochloride is not optically active.

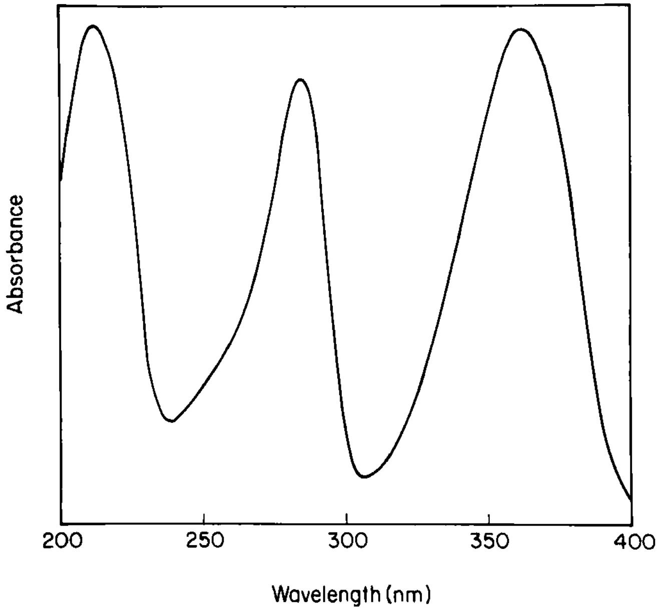


Figure 6. Ultraviolet spectrum of a solution of amiloride hydrochloride in 0.1N HCl.

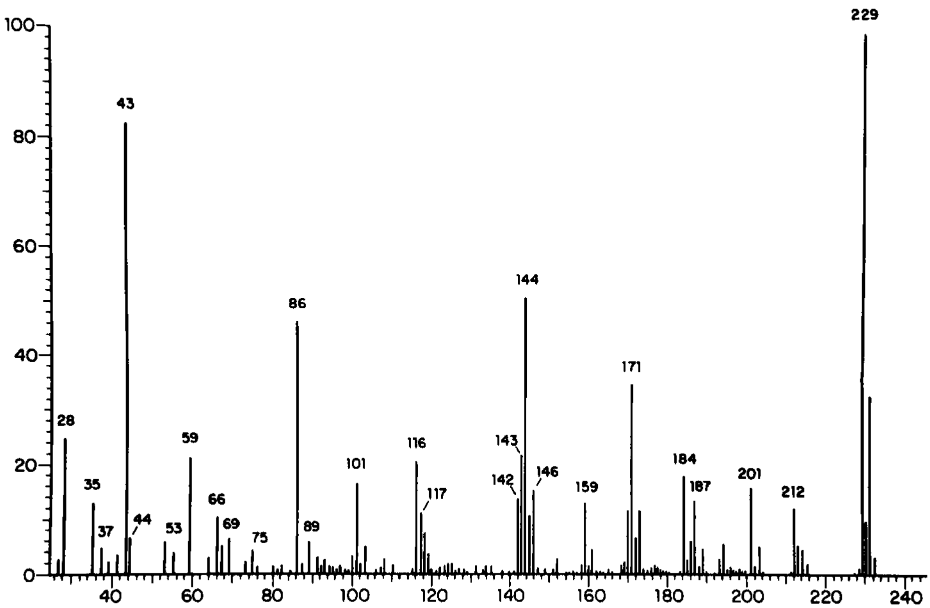


Figure 7. Low resolution mass spectrum of amiloride hydrochloride taken in the electron impact mode.

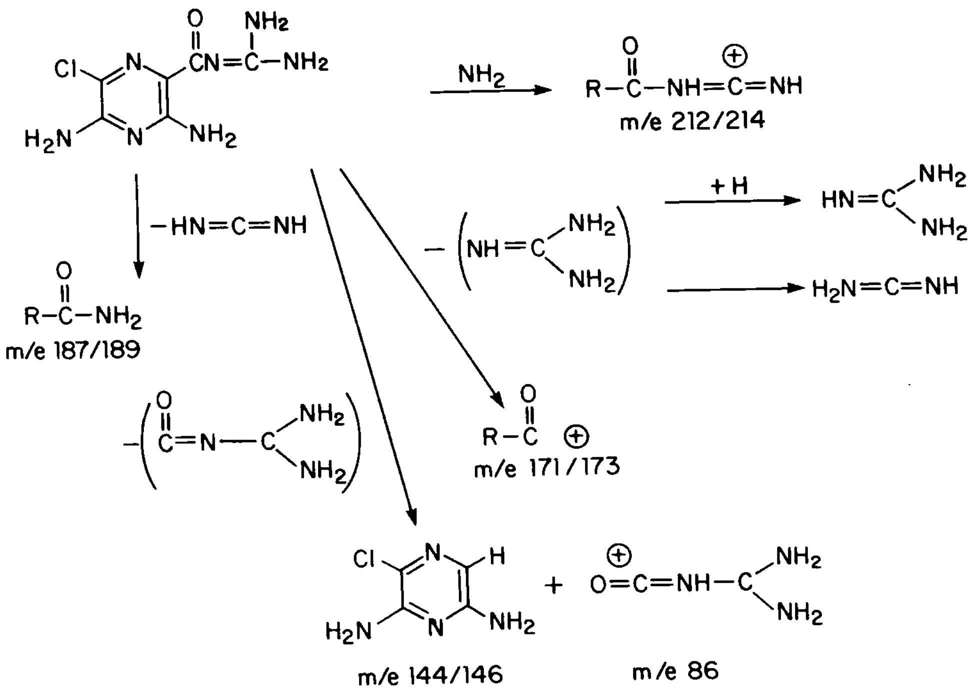


Figure 8. Schematic representation of the mass spectral fragmentation of amiloride hydrochloride.

4.6 Thermoanalytical Behavior

4.6.1 Melting Point

The melting point (with decomposition) of anhydrous amiloride hydrochloride is 293.5°C. The dihydrate melts with decomposition at approximately 288°C (20).

4.6.2 Differential Thermal Analysis Behavior (17)

The differential thermal analysis (DTA) curve of amiloride hydrochloride is characterized by a water loss endotherm with a peak temperature of ca. 136°C. An endotherm-exotherm combination is observed at ca. 300°C. Actual peak temperatures are 298°C and 304°C for the endotherm and exotherm respectively. This thermal event corresponds to a melting plus decomposition.

4.6.3 Thermogravimetric Analysis Behavior (17)

Thermogravimetric analysis of amiloride hydrochloride indicates no weight loss until ~ 90°C. From ~ 90°C to ~ 170°C, there is an approximately 12% weight loss due to dehydration of the dihydrate. No further weight loss occurs until sublimation/decomposition begins at ~ 280°C.

4.7 Solubility

The solubility of amiloride hydrochloride in a variety of solvents at room temperature (~ 25°C) is presented in Table IV (18). Note that these solubilities are stated in terms of the current U.S.P. definitions (21).

Table IV
Solubility of Amiloride Hydrochloride at Room Temperature

Solvent	Solubility
Acetone	Practically insoluble
Chloroform	Practically insoluble
Diethylether	Practically insoluble
Dimethylsulfoxide	Freely soluble
Ethanol	Very slightly soluble
Ethylacetate	Practically insoluble
Isopropanol	Slightly soluble
Methanol	Sparingly soluble
Water	Slightly soluble

The solubility of amiloride hydrochloride in water is typical of an organic base with limited aqueous solubility and increases with a decrease in pH (Table V) (18).

Table V

Aqueous Solubility of Amiloride Hydrochloride As a Function of pH

pH	Solubility (mg/mL)
4.8	5.2
7.6	5.1
9.4	0.5
10.0	0.3

4.8 Crystal Properties

Amiloride hydrochloride dihydrate exists as a crystalline powder. Variations encountered in the X-ray powder diffraction pattern suggest the existence of at least two polymorphic forms (22). Polymorphism of amiloride hydrochloride has not been detected by other physical and/or chemical measurement techniques. Figures 9 and 10 show the X-ray powder diffraction patterns for the two

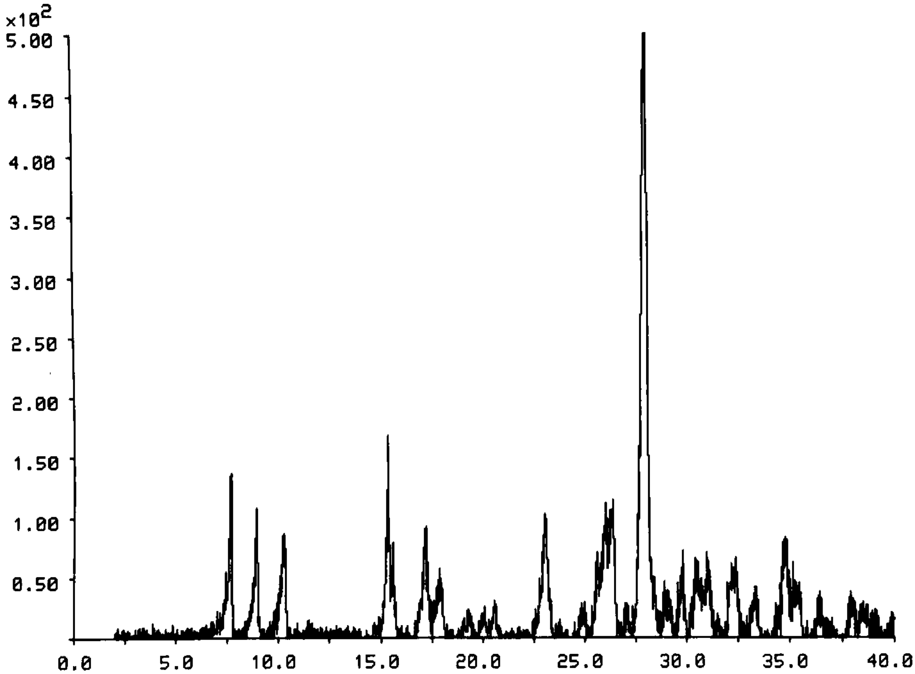


Figure 9. X-ray powder diffraction pattern of polymorph A of amiloride hydrochloride.

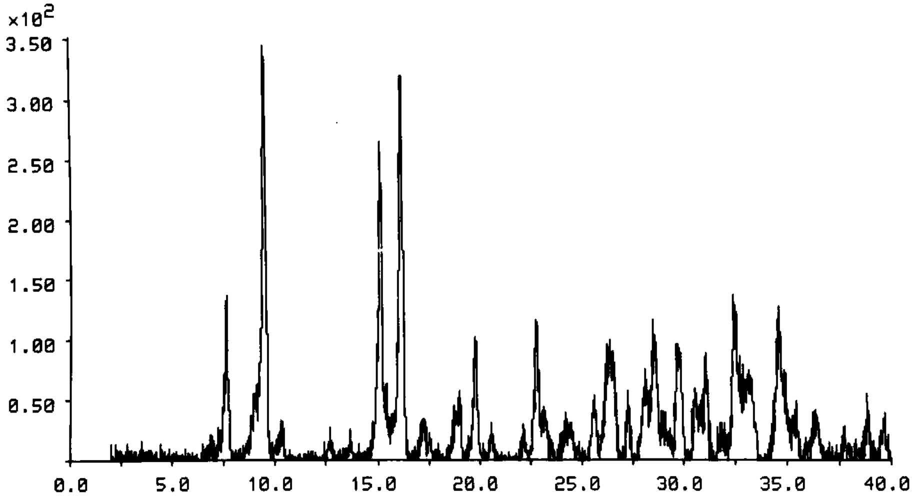


Figure 10. X-ray powder diffraction pattern of polymorph B of amiloride hydrochloride.

polymorphs (A and B) seen to date. The patterns were obtained using a Phillips Electronics model APD3720 powder diffractometer using copper Kα radiation.

Tables VI and VII list the interplanar distances and the relative intensities of the major lines in the X-ray powder diffraction patterns of polymorphs A and B, respectively.

Table VI

X-Ray Powder Diffraction of Amiloride Hydrochloride Polymorph A

Peak Angle (2θ)	I/Imax (%)
7.6725	29.03
8.9150	22.55
10.2425	15.06
12.8400	2.14
15.3800	30.02
15.6100	14.71
17.1975	16.89
17.8950	7.76
19.3425	4.41
20.0200	4.22
20.5925	6.08
23.1600	20.85
24.9000	5.86
25.6375	15.42
26.0275	23.43
26.4375	23.87
27.0325	5.86
28.0650	100.0
29.1425	7.51
29.8300	14.02
30.4700	11.11
31.0600	10.22
32.4275	9.08
33.3500	5.64
34.8350	20.44
35.4775	12.04
36.4200	4.61
37.9675	5.01

Table VII

X-Ray Powder Diffraction of
Amiloride Hydrochloride Polymorph B

Peak Angle (2θ)	I/Imax (%)
6.7350	6.49
7.5025	31.95
8.8375	14.36
9.4075	100.00
10.2150	7.13
12.5850	5.57
13.6050	5.57
15.0775	59.32
15.3400	14.83
16.1350	73.47
17.1500	10.43
18.6225	11.67
18.9125	17.84
19.7450	25.31
20.4850	5.87
22.1000	7.47
22.7050	29.20
23.1300	13.43
24.2150	14.83
25.4975	23.47
26.2200	63.21
26.5000	65.20
27.1625	14.36
28.0425	44.17
28.4525	42.53
29.7150	40.14
30.4400	14.36
31.0225	12.10
31.7100	7.13
32.4225	34.09
33.2050	19.45
34.5275	37.81
35.2825	12.98
36.2325	7.47
36.4600	3.95
37.7200	6.17
38.7850	10.84

4.9 Hygroscopicity

The monohydrochloride salt of amiloride forms a stable dihydrate and amiloride hydrochloride, unless stated otherwise, is supplied as such. Amiloride monohydrochloride dihydrate may be converted to the anhydrous form of the salt by drying at 100°C at pressures $\leq$ 6.6 x 10^{-3} atm for 3 hours (23). No other stable hydrates of amiloride hydrochloride have been reported (24).

4.10 Dissociation Constant

The dissociation constant of amiloride hydrochloride as derived from aqueous titration (18) indicates that amiloride is a moderately strong organic base with a pKa of approximately 8.7 at 25°C (amidino nitrogen).

The pKa of amiloride hydrochloride and, in fact, many pyrazinylguanidine derivatives has also been determined using gas-phase proton affinities, enthalpies of solution and semi-empirical calculations (25, 26). These theoretically derived pKa values agree well with the experimentally determined value of pKa = 8.7.

5. Methods of Analysis

5.1 Identification Tests

5.1.1 Ultraviolet Spectrophotometry

Ultraviolet spectrophotometry is used to identify amiloride hydrochloride. A solution in 0.1 N HCl scanned from 200 nm to 400 nm qualitatively exhibits the same absorbance characteristics at identical wavelengths as does a similarly prepared and concomitantly measured solution of an amiloride hydrochloride standard. Quantitatively, equimolar sample and standard solutions will exhibit absorbances at ~ 360 nm (λ_{max}) which differ by no more than 3%.

5.1.2 Infrared Spectroscopy

Infrared spectroscopy may also be used to identify amiloride hydrochloride. The infrared absorption spectrum prepared as a potassium bromide disk or a mineral oil dispersion compares qualitatively (with maxima only at the same frequencies) to the spectrum of a similarly prepared amiloride hydrochloride standard.

5.1.3 Elemental Analysis

Elemental analysis has been employed to identify amiloride hydrochloride. The results of a weight percent determination of carbon, nitrogen, hydrogen and chloride are compared to the respective theoretical values of 28.85%, 32.45%, 4.34% and 23.47%. Amiloride hydrochloride will respond positively to the test for chloride described in the United States Pharmacopeia (27).

5.2 Spectrophotometric Analysis

5.2.1 Direct Ultraviolet Spectrophotometry

Amiloride hydrochloride exhibits a UV absorption band near 360 nm attributed to the substituted pyrazine ring system. This absorption is the basis for the quantitative determination of the drug. Assay of the compound is based on a comparison of the net absorbance at 360 nm of a sample in 0.1 N HCl with a standard in 0.1 N HCl of known concentration. The net absorbance is calculated by substracting the drug free matrix contribution to absorbance at the wavelength of determination from the absorbance of the drug solution at the same wavelength.

5.2.2 Ultraviolet Spectrophotometry Via Flow Injection Analysis

Ultraviolet absorbance at 360 nm is also used as the detection mode for Amiloride hydrochloride determinations by flow injection analysis (FIA) (28). In the case of FIA assay, amiloride hydrochloride sample and standard solutions are periodically injected into a flowing stream. The resulting changes in UV absorbance at 360 nm of the stream are measured relative to the drug free stream. Calculation of the concentration of amiloride is made in an identical fashion as direct UV spectrophotometry.

5.2.3 Liquid-Liquid Extraction with Ultraviolet Spectrophotometry or Spectrofluorimetry

Amiloride hydrochloride may be determined in the presence of its degradation products by a liquid-liquid extraction technique followed by quantitation by ultraviolet spectrophotometry or spectrofluorimetry (18). An alkaline aqueous solution of amiloride hydrochloride is extracted with tributyl phosphate. Amiloride is partitioned into the organic layer while its degradates remain in the aqueous phase. Amiloride is then determined by ultraviolet spectrophotometry at 360 nm or fluorimetry with an excitation wavelength of ca. 360 nm and an emission wavelength of ca. 420 nm. Sensitivity is ~ 0.2 ppm.

5.3 Chromatographic Analysis

5.3.1 Thin Layer Chromatography

Normal-phase thin layer chromatography on silica gel using one of two developing solvent systems has been employed for amiloride hydrochloride. In the first system, a developing solvent of 10% n-propanol in chloroform is used to develop a spot resulting from 5 µL of a 1% aqueous solution of

the drug. R_f for the analyte in this system is approximately zero (R_f ~ 0). Detection is by UV absorbance at 254 nm or 360 nm (most sensitive). A sensitivity of 0.01% has been reported. In the second TLC system, 4 parts 3 N aqueous ammonium hydroxide are mixed with 30 parts of tetrahydrofuran to form the developing solvent (23). A 1 µL spot of a 0.1% aqueous solution of the drug is developed and detected by UV absorbance at 254 nm or 360 nm. R_f for the analyte is approximately 0.7. Sensitivity under these conditions is in the range of 0.1% to 0.5% of the analyte concentration.

5.3.2 High Performance Liquid Chromatography

Reversed-phase HPLC is routinely used to determine amiloride hydrochloride (29). The method employs an ES Industries C-2(300 mm x 4.6 mm i.d., 10 µm particle size) HPLC column operated at ambient room temperature (~ 25°C). A mobile phase consisting of 85% aqueous 0.01M sodium hexane sulfonate (pH = 3.0) in acetonitrile is used to elute amiloride. Flow rate is 2.0 mL/min and detection is by UV absorbance at 280 nm. Under these conditions, amiloride elutes in less than 8 minutes (k' ~ 2). Typically 20 µL injections of an approximately 200 µg/mL drug solution are made.

5.4 Non-Aqueous Titration

Amiloride hydrochloride can be determined by non-aqueous titration with perchloric acid (23). The assay involves dissolution of an appropriate amount (~ 450 mg) of amiloride hydrochloride in 100 mL of glacial acetic acid to which is then added 10 mL of mercuric acetate, 15 mL of dioxane and an appropriate amount of crystal violet indicator. The well mixed solution is titrated with 0.1N perchloric acid to a blue endpoint. Assay results must be corrected for a blank titration. Each equivalent of perchloric acid is equivalent to 26.61 mg of amiloride hydrochloride.

6. Stability - Degradation

6.1 Solid State Stability

Amiloride hydrochloride is a stable compound at room temperature in the solid state, i.e. no significant degradation has been observed in samples exposed to no excessive humidity for seven years. Some darkening of amiloride hydrochloride powder has been observed upon exposure to intense ultraviolet light (24 times the strength of direct sunlight) but no degradation products were detected by thin layer chromatography, even after 7 days of exposure (18). Elevated temperatures as high as 100°C for 1 week in the absence of excessive humidity do not produce degradation. Only prolonged (> 1 week) exposure to very high humidity (> 90% RH) and temperatures greater than 60°C will produce significant degradation in the solid state.

6.2 Solution Stability

Aqueous solutions of amiloride hydrochloride are stable at usual ambient temperatures. A study of the stability of amiloride hydrochloride in aqueous solution at elevated temperatures at various pH levels resulted in the identification of three degradation products (Figure 11). The relative amounts of the three degradates vary with pH. At pH < 1, compound I dominates, at pH ~5 both II and III are present and in alkaline solution (pH > 13), compound III predominates. All three potential degradation products are acidic in comparison to the parent compound. The degradation of amiloride hydrochloride in alkaline solution (pH > 13) follows first-order degradation kinetics (18). The chemical transformation of amiloride hydrochloride in solution formulation with other pharmaceutical agents and excipients has been shown to be more complex than that observed in simple aqueous solution (30).

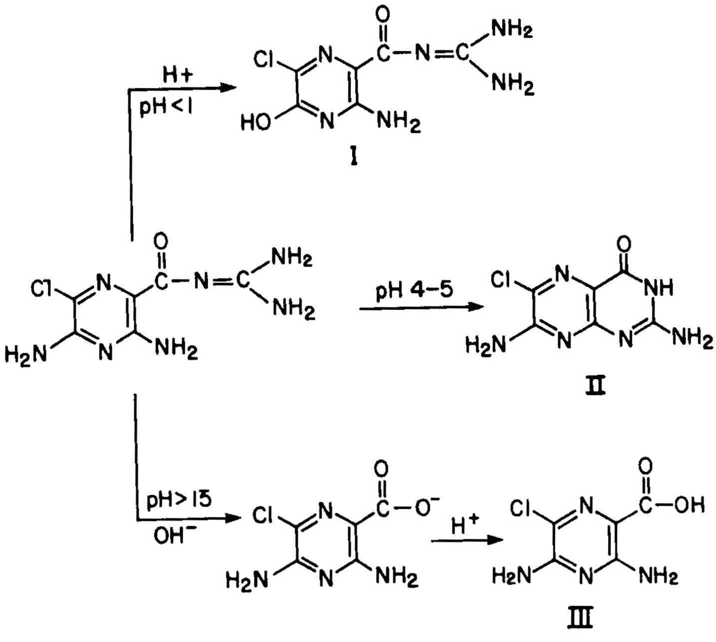


Figure 11. Solution degradation products of amiloride hydrochloride.

7. Biopharmaceutics and Metabolism

7.1 Absorption and Bioavailability

It has been shown, using radiolabelled (C^{14}) amiloride hydrochloride, that approximately 50% of an oral dose administered to man is absorbed from the gastrointestinal tract (31,32). The remainder of the dose is unabsorbed and can be found in the feces (31-34). Human bioavailability studies comparing 5 mg tablets of amiloride hydrochloride to an aqueous reference solution showed that the pharmaceutical dosage form was fully bioequivalent (33). Absorption of amiloride hydrochloride is reduced when administered with food (33,25).

7.2 Metabolism

Amiloride hydrochloride which has been absorbed in man is excreted without metabolism in the urine (31,33,34,36). No metabolic products have been detected and/or identified. Since the drug is not metabolized it probably can be administered to patients with hepatic dysfunction, providing that renal excretory function is normal (34). Amiloride hydrochloride is cleared from the body by tubular transport (5,7,31-34,36) and, in combination with hydrochlorothiazide, does not alter the normal renal potassium excretion or the rate of urinary variable excretion (37).

7.3 Pharmacokinetics

Amiloride hydrochloride is rapidly absorbed from the gastrointestinal tract (31,32,38-40). Onset of the physiological effect of the drug in man is usually noted within two hours (34,36) with peak serum levels being achieved in between 3 and 4 hours (31,32). Typical pharmacokinetic half-life values ($T_{1/2}$) range between 6 and 10 hours (31,32,36). Considerable prolongation of the half-life has been noted in patients with chronic renal failure (33,34,36,39). Effects of the drug generally subside within 24 hours resulting in urinary levels of less than 0.5% of the administered dose (31,36). Urinary amiloride concentrations range from 4 μg/mL to 30 μg/mL during diuresis (31,32) with peak renal clearance of

amiloride ranging from 400 mL/min to 600 mL/min (33). A dose-response relationship has been observed in man (33,34,36). Progressively increasing effects of single doses have been noted from 1 mg to 40 mg with a plateau being reached above 40 mg.

8. Determination in Biological Matrices

Several techniques have been used to determine amiloride in biological fluids. Among them, liquid scintillation counting for radiolabelled compound, liquid-liquid extraction with measurement of ultraviolet absorbance or fluorescence, thin-layer chromatography with UV or fluorescence detection and high performance liquid chromatography (HPLC) with fluorescence detection have been the most widely used.

Liquid scintillation counting of C^{14} labelled amiloride in urine, serum, plasma, tissue and feces has been performed routinely (31,32,41) usually with an internal standard used for quantitation of the analyte.

Liquid-liquid extraction of amiloride from serum, plasma and/or urine has been performed with a 7% saturated aqueous sodium carbonate solution in ethyl acetate (1 part drug containing fluid to 27 parts extracting solution). The analyte is back-extracted from the ethyl acetate phase into 0.1 N HCl and then determined by spectrofluorimetry (λ_{excite} = 365 nm, $\lambda_{emit.}$ = 420 nm) (40). In matrices containing minimal interferents, ultraviolet absorbance at 360 nm may also be used.

Normal-phase thin-layer chromatography (TLC) on silica has also been employed for the determination of amiloride from biological fluids (31,41,42). Generally, an extraction technique similar to that described above is employed for drug isolation prior to TLC. A common developing solvent consists of butanol-acetic acid-water (100:27:73) (41). Other extraction techniques and developing solvents have been reported (42). Detection of TLC spots is accomplished by fluorescence with long wavelength UV excitation and emission at ca. 425 nm.

Finally, reversed-phase high performance liquid chromatography with fluorescence detection has become popular for the determination of amiloride in biological fluids. Two HPLC systems have been reported (43,44) with large numbers of samples being processed by one of these systems (44). A C_{18} HPLC column (300 mm x 3.9 mm i.d., 10 µm particle size) is employed with a mobile phase of 60:40 [0.1M $NaPO_4$(pH = 4.0)]:CH_3OH flowing at 1.0 mL/min. Column temperature is ~ 25°C and detection is by fluorescence with an excitation wavelength of 368 nm and an emission wavelength of 417 nm. Under these conditions, amiloride elutes in ~ 6 minutes. Quantitation is performed using an internal standard, 3,5-diamino-N-(aminoiminomethyl)-6-fluoropyrazinecarboxamide.

9. Determination in Pharmaceuticals

9.1 Dissolution Testing

The determination of amiloride hydrochloride in samples resulting from dissolution testing of tablets containing the drug can be accomplished by ultraviolet absorbance at 360 nm. Either direct UV spectrophotometry or UV spectrophotometry vis-a-vis flow injection analysis may be used (45).

9.2 Assay, Dosage Uniformity and Stability Testing

High performance liquid chromatography is the technique of choice for the determination of amiloride hydrochloride in tablets for release and/or stability purposes although ultraviolet spectrophotometry may be used for the determination of dosage uniformity. Reversed-phase HPLC with UV detection at 360 nm has been performed using a C_{18} HPLC column (300 mm x 3.9 mm i.d., 10 µm particle size) with a mobile phase of 25% methanol in 0.05 M phosphate buffer (pH = 3.0). Flow rate was 1.0 mL/min (30). Recently, a fast-HPLC method was developed (46) which employs a C_{18} mini-column (50 mm x 4.6 mm i.d., 5 µm particle size). Using a mobile phase of 20% methanol in 0.02M phosphate buffer pH = 2 at a

flow rate of 4.0 mL/min amiloride is eluted in approximately 0.5 minutes (k' ~ 1). Detection is again by UV absorbance at 360 nm. This method, because of its speed, has also been used routinely for dosage uniformity samples.

Acknowledgement

Special thanks go to Mrs. Elizabeth Moyer for typing the manuscript. Thanks are also in order to Mr. James Ryan and Dr. James McCauley for obtaining the IR spectra and X-ray diffraction patterns, respectively. The assistance of Ms. Florence Berg for literature searching and Mr. William Shearin for background information retrieval is also acknowledged. Finally, sincere gratitude is expressed to Dr. Gerald Brenner for his review of the manuscript.

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Chemical Abstracts was searched for amiloride hydrochloride citations from 1966 to 1985.

AMINOGLUTETHIMIDE

HASSAN Y ABOUL-ENEIN

ANALYTICAL PROFILE

1. Description

1.1 Introduction:

Aminoglutethimide was initially developed as an anticonvulsant in the 1950's but was later withdrawn from clinical use after reports of adrenal insufficiency (1). It was subsequently shown to suppress adrenal steroid synthesis by inhibiting the demolase enzyme system which is responsible for the conversion of cholesterol to Δ^5 -pregnenolone (2). It has been used in the treatment of adrenocortical tumours and Cushing's syndrome (3).

Aminoglutethimide inhibits steroid aromatase enzyme involved in the biosynthesis of estrogens (4), e.g. conversion of androstenedione to esterone. It is currently used as an effective agent for the treatment of advanced breast cancer in post-menopausal women (5,6,7).

1.2 Nomenclature:

1.2.1 Chemical Names:

α - (p-Aminophenyl) - α - Ethylglutarimide

2 - (p-Aminophenyl) - 2 - Ethylglutarimide

3 - (4-Aminophenyl)- 3 - Ethyl - 2,6 - piperidinedione

3 - Ethyl - 3 - (*p.* aminophenyl) - 2,6 - dioxopiperidine

1.2.2 Generic Names:

Aminoglutethimide, Ciba 16038

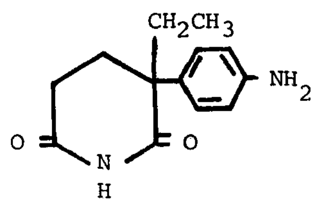
1.2.3 Trade Names:

Elipten, Cytadren, Orimeten

1.3 Formula

1.3.1 Empirical: $C_{13}H_{16}N_2O_2$

1.3.2 Structural :



1.3.3 Chemical Abstract Registry Number: 125-84-8

1.4 Molecular Weight: 232.27

1.5 Elemental Composition: C 67.22%; H 6.94%; N 12.06%; O 13.78%

1.6 Appearance, Color, Odor and Taste: a white to creamy white crystalline powder, odorless and possessing a bitter taste (8,9,10).

1.7 pH (0.1% solution): 6.2-7.3

Introduce 200.0 mg $\pm$ 0.2 mg of aminoglutethimide into a 200 ml volumetric flask, dissolve in 10.0 ml of methanol and add approx. 180 ml of CO_2-free water

at 50°C. After shaking well, cool down to room temperature and fill up to the mark with CO_2-free water at room temperature, then determine the pH potentiometrically (8).

2. Physical Properties

2.1 Melting Range:

The following melting range has been reported for aminoglutethimide:

M.p, C°	Reference
151	9
149-150 (from MeOH or EtOAc)	10
152-154	11

The hydrochloride salt [CAS 31075-85-1] has a melting range of 223-225° (9).

2.2 Solubility:

Virtually insoluble in water, freely soluble in most organic solvents e.g. methanol, methylene chloride and chloroform. Poorly soluble in ethylacetate, 0.1N HCl and absolute ethanol, readily soluble in acetone and 100% acetic acid (8,9).

2.3 Dipole Moment:

Lee and Kumler(12) determined the dipole moment and structure of the imide group in five and six membered cyclic imides. Aminoglutethimide has a dipole moment of 2.83 μ in D unit determined at 30° in dioxane.

2.4 Optical Activity and Absolute Configurations:

Finch et al (13) resolved the optical enantiomers of aminoglutethimide via recrystallization of the tartarate salt from methanol. The dextrorotatory antipode (+) aminoglutethimide has a m.p. 114-115° and $[\alpha]_D^{25}$ = + 163.1 (MeOH).

The levorotatory antipode (-) aminoglutethimide(II) has a m.p. 114-115° and $[\alpha]_D^{25}$ = -163.6° (MeOH). The absolute configuration of (+) - isomer was determined to have R-configuration while (-) - isomer has S-configuration around the assymmetric carbon as shown in Figure 1. It is of interest to mention that the (+)-isomer (I) had the most of the steroid synthesis inhibition activity (2-3 more potent than the racemate), while the (-) isomer had very little activity at dose levels 10-fold higher.

2.5 Spectral Properties

2.5.1 Ultraviolet Spectrum

The ultraviolet spectrum of aminoglutethimide in neutral methanol is shown in Fig. 2. It exhibits a maximum at about 242 nm and a shoulder at approximately 282 nm. The maxima at 242 and 282 nm do not change or shift in acidic (0.1N H_2SO_4) or basic (0.1N NaOH) media as shown in Figures 3 and 4. The ultraviolet

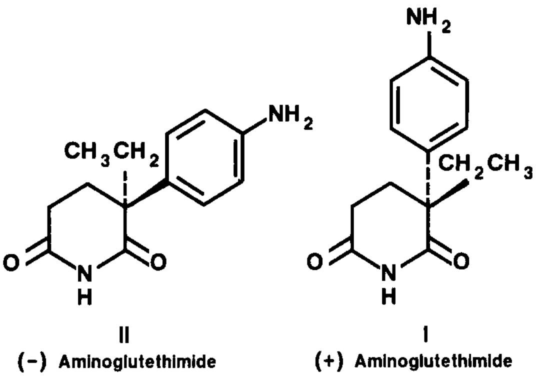


Fig. 1 - The absolute configuration of (-) - S and (+) - R - Aminoglutethimide

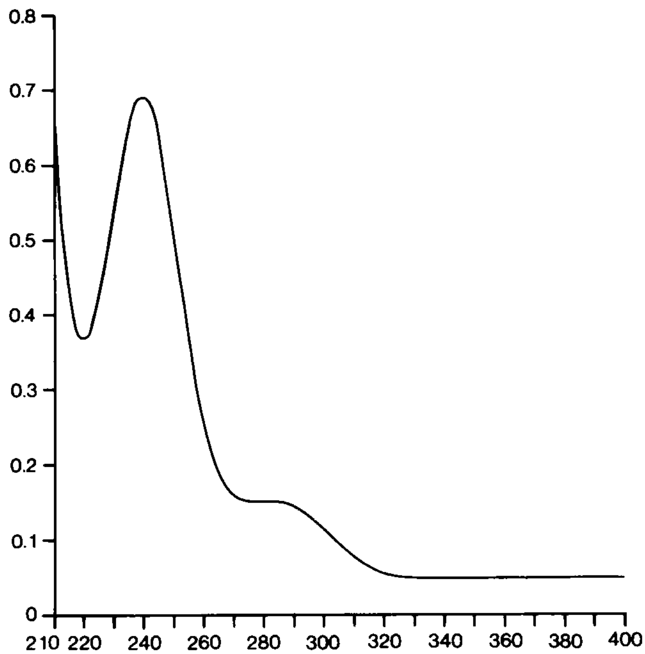


Fig. 2 - Ultraviolet spectrum of aminoglutethimide in neutral methanol.

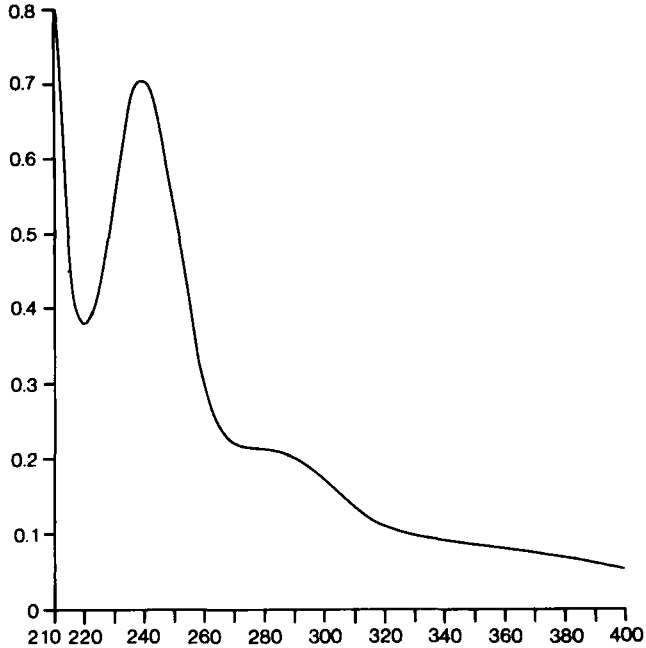


Fig 4. - Ultraviolet spectrum of aminoglutethimide in 0.1N NaOH.

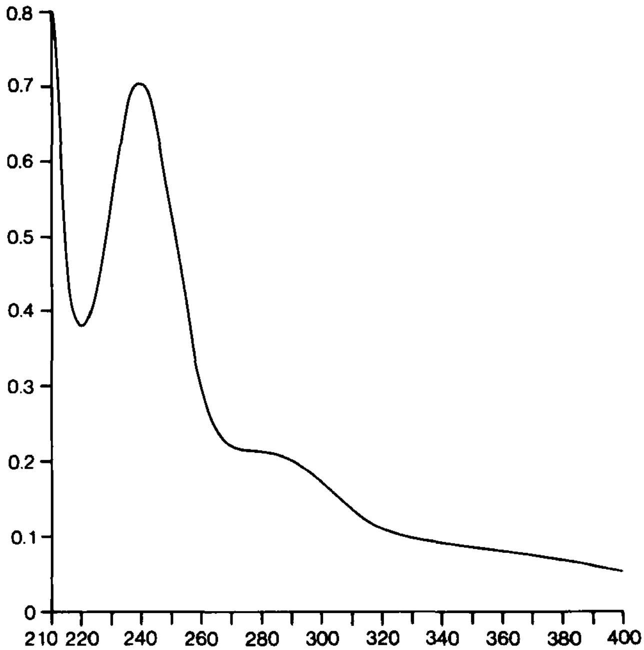


Fig. 3 - Ultraviolet spectrum of aminoglutethimide in $0.1NH_2SO_4$.

spectra are recorded on a Varian AG UV-VIS spectrophotometer - Model DMS-90. These data are in agreement with previously published information (14).

2.5.2 Infrared Spectrum

The infrared of aminoglutethimide in Nujol mull is presented in Figure 5 and is recorded on Perkin-Elmer spectrophotometer model 580B. The frequencies and their structural assignments are as follows:

Frequency (cm^{-1})	Assignment
3470, 3480	ν asym NH_2 stretching vibration
3180	ν sym-NH-imide group
3080	CH aromatic stretch
1715, 1690	C=O imide carbonyl stretch of glutarimide ring
1630, 1520	C=C aromatic for phenyl skeletal vibrations
830 & 700	CH-out-of-plane bending vibration characteristic for p-substitution

Other characteristic finger print bands are 1260, 1200 cm^{-1}. These data are in agreement with previously reported reports (8,14).

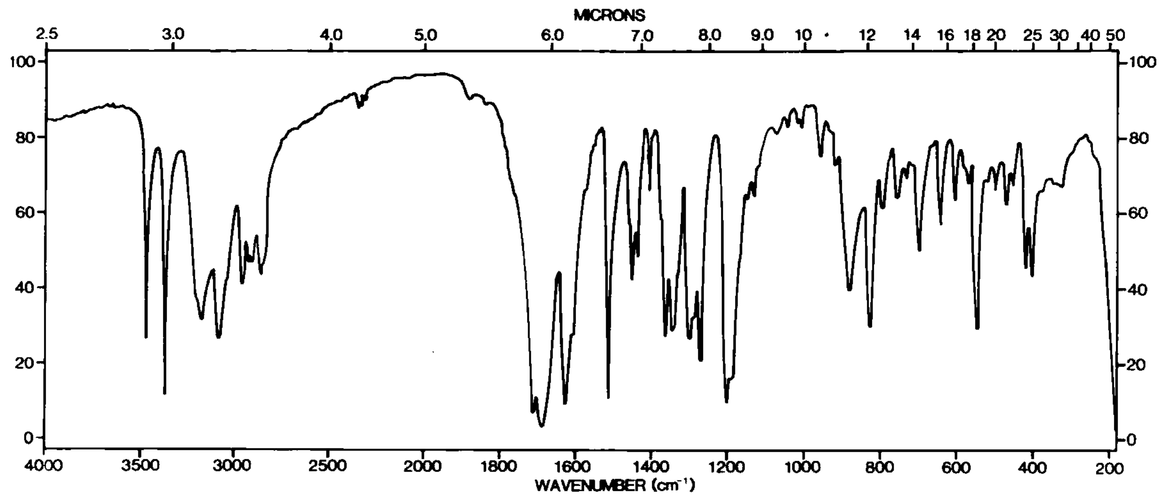


Fig. 5 - Infrared spectrum of aminoglutethimide in Nujol mull.

2.5.3 Nuclear Magnetic Resonance Spectra

2.5.3.1 PMR Spectrum

The 60MHz PMR spectrum of aminoglutethimide in DMSO-d_6 is shown in Figure 6. The spectrum was recorded on a Varian T_{60}-A NMR spectrometer using TMS as the internal standard. The following structural assignments have been elicited from Figure 6:

Chemical Shift (ppm)	Assignments
0.77 t	$\underline{CH_3}-CH_2$
1.80 q	CH_3-CH_2
2.27 m	CH_2-CH_2-CH of the glutarimide ring system
6.53 d J=8.5 Hz	aromatic H_3 & H_5
6.95 d J=8.5 Hz	aromatic H_2 & H_6
	AB system characteristic of phenyl p-substitution
5.0 s	NH_2 exchangeable with D_2O
9.33 s	NH exchangeable with D_2O

s=singlet, d=doublet, m-multiplet, q=quartet t=triplet

Defay and Dorlet (15) reported the assignments of NMR spectra of some glutarimide derivatives including

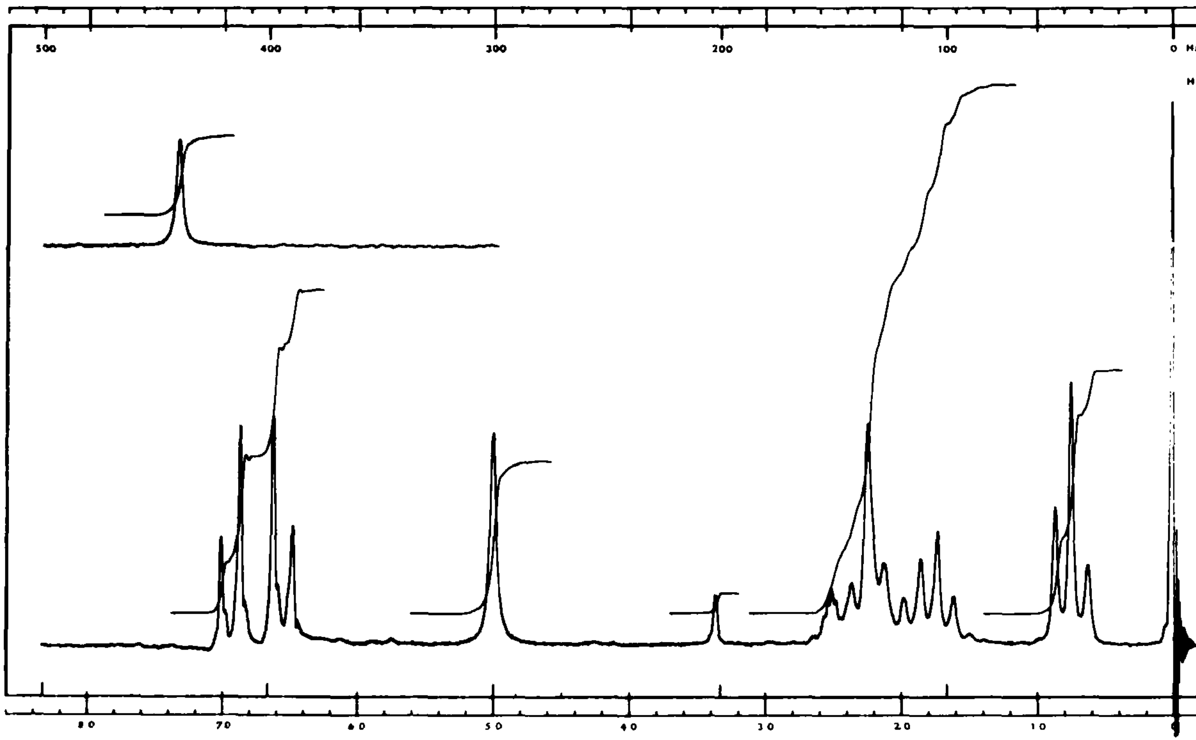
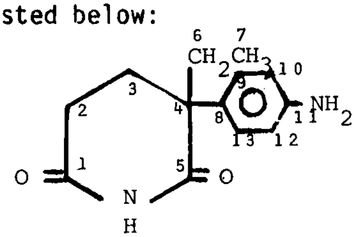


Fig. 6 - PMR spectrum of aminoglutethimide in DMSO - d_6 using TMS as the internal standard.

aminoglutethimide and is in agreement with the assigned peaks.

2.5.3.2 ^{13}C-NMR Spectrum

The ^{13}C-NMR spectrum of aminoglutethimide has been determined on a Varian FT80 spectrometer at ambient temperature. The sample was dissolved in DMSO-d_6 in a tube with 10mm diameter. Spectral width: 5000 Hz, acquisition time: 1.638 seconds, pulse width 6 μ second and number of data points 16384. The noise decoupled and the complete off-resonance spectra are shown in Figures 7 and 8, respectively. The spectral assignments are listed below:



Chemical Shift (in ppm relative to TMS)	Assignment
176.23 s	C=O at C_5
172.92 s	C=O at C_1
147.46 s	C_{11}
126.66 d	C_9 & C_{13}

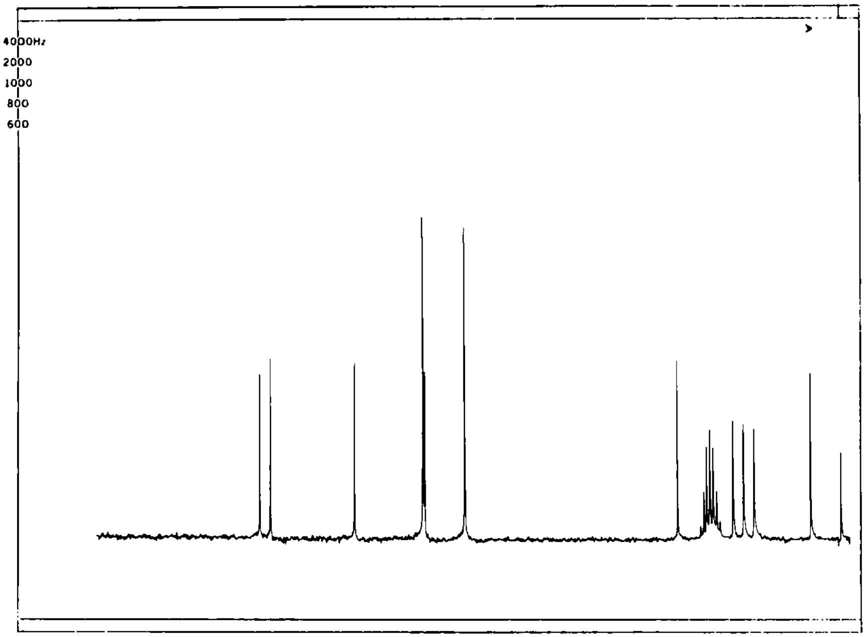


Fig. 7 - 13C-NMR off-resonance spectrum of aminoglutethimide in DMSO-d_6.

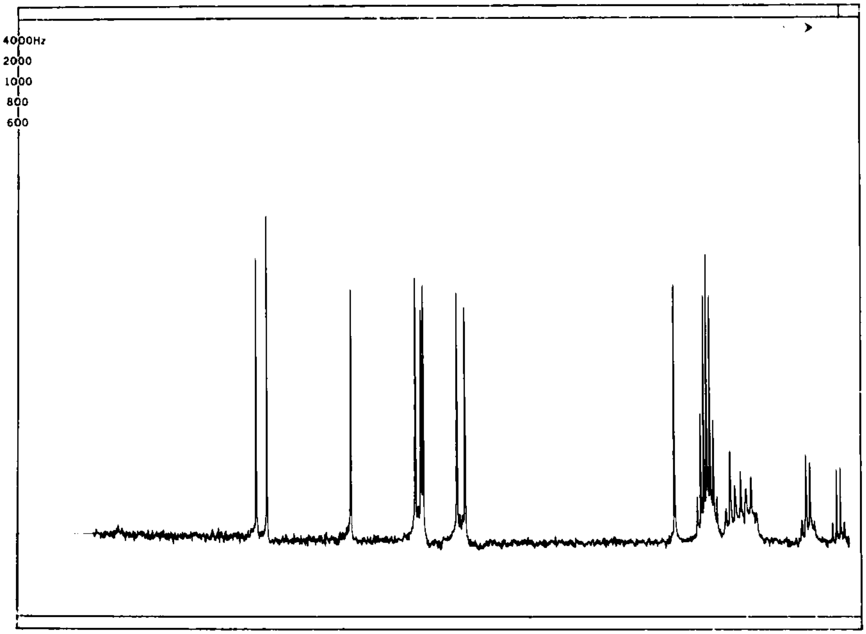


Fig. 8 - $_{13}$C-MNR off-resonance spectrum of aminoglutethimde in DMSO-d_6.

126.11 s	C_8
114.04 d	C_{10} & C_{12}
49.27 s	C_4
32.35 t	C_3
29.17 t	C_2
26.04 t	C_6
8.86 q	C_7

s=singlet, d=doublet, t=triplet, q=quartet

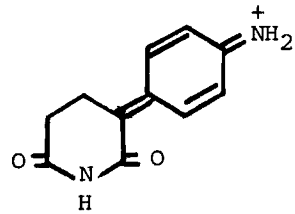
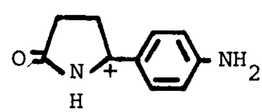
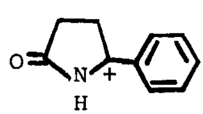
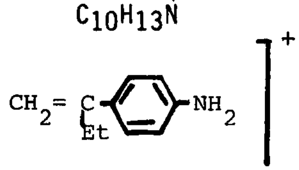
2.5.4 Mass Spectrum

The mass spectrum of aminoglutethimide obtained by chemical ionization (CI) on a solid probe using CH_4 as ionizing gas is shown in Figure 9. The (M + 1) peak is at m/z 233 (100%) which corresponds to its molecular weight, a small peak (M + 29) is also present at m/z 261 (5%). The mass spectrum of aminoglutethimide obtained by helium charge exchange (HCE) with direct inlet probe is shown in Figure 10.

The spectra were recorded on a Finnigan 3200 GC/MS connected to an Incos 2300 data system. The spectrum (Figure 10) shows a molecular ion peak (M^+) as m/z 232 and a base peak at m/z

132. The most prominent fragments are listed in Table 1.

TABLE 1. Most Prominent Fragments of Aminoglutethimide

Mass m/z	Relative Intensity, %	Fragment
232	31.2	$C_{13}H_{16}N_2O^{+}{}_{2}$, M^+ 
203	45.2	$C_{11}H_{11}N_2\overset{+}{O}_2$, $M-C_2\overset{+}{H}_5$
175	34.4	$C_{10}H_{11}N_2\overset{+}{O}$ 
160	14	$C_{10}H_{10}N\overset{+}{O}$ 
147	11.4	$C_{10}H_{13}\overset{+}{N}$ 

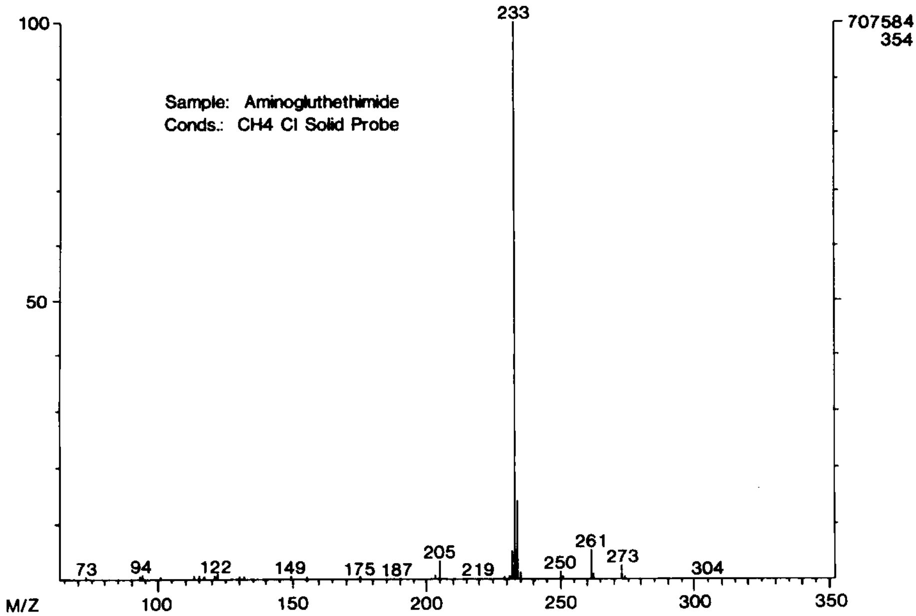


Fig. 9 - Chemical ionization (CI) mass spectrum of aminoglutethimde using CH_4 as ionizing gas.

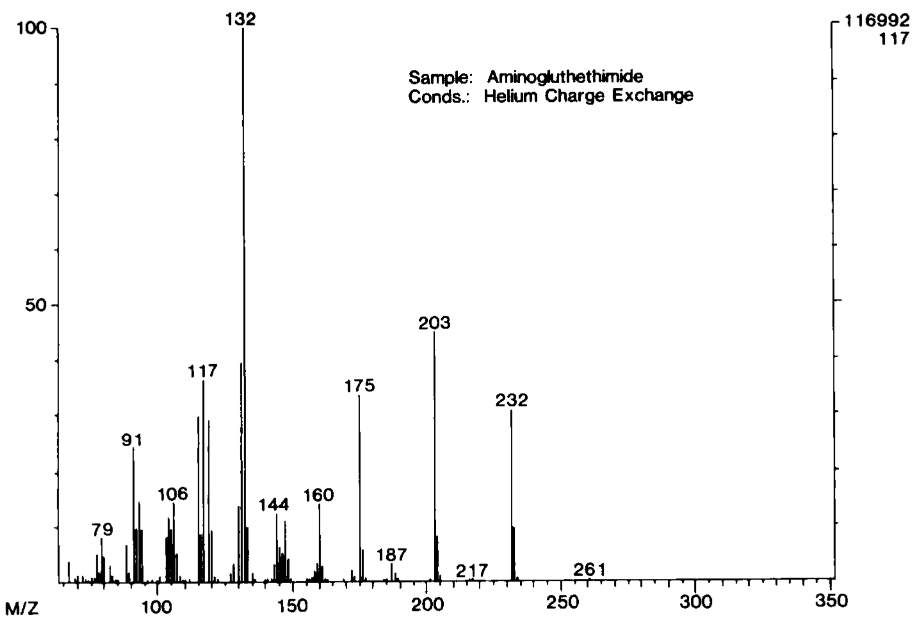
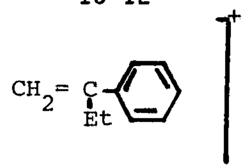
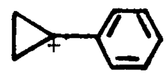


Fig. 10 - Helium charge exchange (HCE) mass spectrum of aminoglutethimide by direct inlet probe.

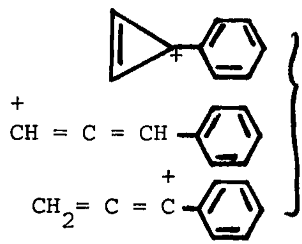
132	100	$C_{10}H_{12}^{+}$



117	37.6	$C_9H_9^{+}$



115	30.5	$C_9H_7^{+}$



Other peaks appear at m/z 131 and 130.

Rücker and Bohn discussed the fragmentation process of glutethimide and aminoglutethimide using the deutrium labeled analogs of these drugs to confirm the structure of the fragments (16).

3. Synthesis

Aminoglutethimide is synthesized as shown in scheme 1 (17). Nitration of 2-phenyl butyronitrile gives the p-nitro derivative. Conjugate Michael addition of the carbanion of p-nitrophenylbutyronitrile to methyl acrylate gives methyl 4-cyano-4 (p-nitrophenyl) hexanoate. The

latter intermediate is cyclized in the presence of acetic acid and 90% sulfuric acid [similar to the synthesis of glutethimide (18)] to give 2-ethyl-2(p-nitrophenyl) glutarimide. Catalytic reduction of the nitro group affords ($\pm$) aminoglutethimide (17).

4. Metabolism

After an oral dose of 250 or 500 mg of aminoglutethimide in man, the drug is absorbed and excreted unchanged to a considerable extent in the urine after 48 hours (19), along with the N-acetyl aminoglutethimide which is considered the major metabolite (20). It has been suggested that this metabolite may contribute to the overall pharmacological activity of the parent drug (20). However, the N-acetyl metabolite has been shown to be less than half as effective in reducing glucocorticoid production as the parent drug (21). Also, it lacked the antifertility activity of aminoglutethimide (22). Sheets and Vickery(23) showed that the ability of aminoglutethimide to inhibit cholesterol conversion to pregnenolone was lost upon acetylation of arylamine nitrogen. The authors explained this fact due to the failure of N-acetylaminoglutethimide to bind to cytochrome P450scc. Aminoglutethimide is among those drugs that are polymorphically acetylated in humans (24). Thus, its effect, as well as some side effects, may be related to the acety-

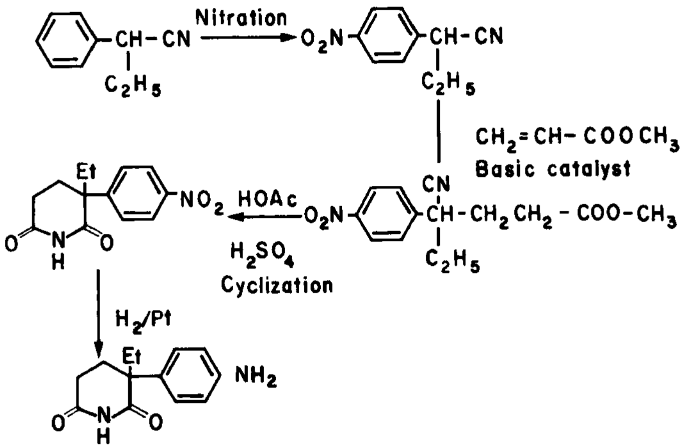
lator phenotype of a particular patient treated with this drug (25). The serum half life was found to be about 7 hours for aminoglutethimide (25). The pharmacokinetics, bioavailability and binding to blood constituents was studied by Thompson et al (26).

Other minor metabolites of the drug have been identified such as N-formylaminoglutethimide and nitroglutethimide (27). N-Hydroxyaminoglutethimide has been recently described as an auto-induced-metabolite that appears in the urine on chronic dosing with aminoglutethimide (28).

The metabolism of aminoglutethimide was studied in the rat by use of the ^{14}C-labeled compound (29). Following oral doses of 5 and 50 mg/kg, the drug was almost completely eliminated within 48 hr into urine and feces, mostly in the form of metabolites. In bile duct-cannulated rats, biliary excretion of radioactivity amounted to about 52% within 24 hr of an orally administered 50 mg/kg dose, with the remainder of the dose being eliminated into urine. The major urinary metabolites resulted from acetylation of the aniline moiety, hydroxylation of the glutarimide ring at positions 3 and 4, and oxidative elimination of the ethyl sidechain. The polar metabolites are accounted for by aromatic hydroxylation with subsequent sulfate conjugation and by a glutarimide ring-opened compound. In

addition, a gamma-butyrolactone derivative was also identified (Figure 11).

Murray _et al_ (30) reported that the half-life of aminoglutethimide administered to patients with mestastatic breast carcinoma was 13.3 $\pm$ 2.65 hours fell significantly to 7.3 $\pm$ 2.14 hours after 6 to 32 weeks of therapy. Recently, four new metabolites of aminoglutethimide have been identified in the urine of patients being treated chronically with the drug (31). These were products of hydroxylation of the glutarimide ring system, namely 3-(4-aminophenyl) -3-ethyl-5-hydroxypiperidine-2, 6-dione and its acetylated analog, 3-(4-aminophenyl) -3-(1-hydroxyethyl) piperidine-2, 6-dione and 3-(4-aminophenyl)3-(2-carboxamido-ethyl) tetra hydrofuran-2-one, a lactone formed by rearrangement of 3-(4-aminophenyl)-3-(2-hydroxyethyl) piperidine -2,6-dione. These metabolites were minor constituents compared with aminoglutethimide and the major metabolites. There were marked species differences between rat and human inasmuch as almost all the urinary metabolites of the rat were N-acetylated whereas most of the human metabolites were not (31). However, 5-hydroxylation occurs in both species, the _cis_ isomer being formed exclusively. All isolated metabolites of aminoglutethimide are biologically inactive as compared to



SCHEME I SYNTHESIS OF AMINOGLUTETHIMIDE

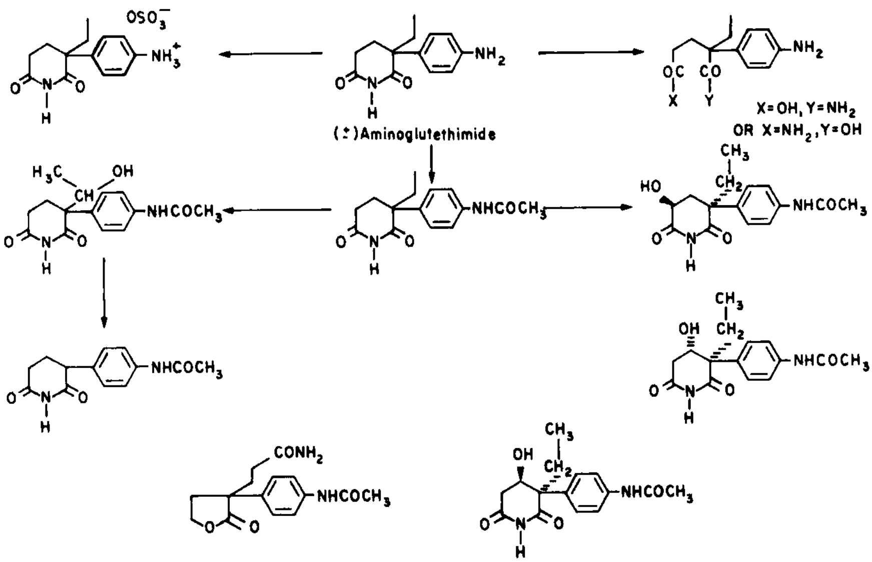
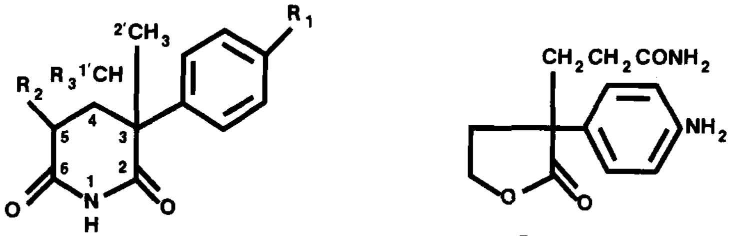


FIG.II PROPOSED METABOLIC PATHWAYS OF AMINOGLUTETHIMIDE IN THE RAT

the parent drug (31,32). The structure of the identified human urinary metabolites of aminoglutethimide are shown in Table 2.

Table 2.. Identified Human Urinary Metabolites of Aminoglutethimide



Compound	R_1	R_2	R_3
Aminoglutethimide	NH_2	H	H
Acetylaminoglutethimide	$NHCOCH_3$	H	H
p-Nitroglutethimide	NO_2	H	H
Formylaminoglutethimide	NHCHO	H	H
Hydroxylaminoglutethimide	NHOH	H	H
p-Amino-5-hydroxyglutethimide	NH_2	OH	H
p-Acetylamino-5-hydroxyglutethimide	$NHCOCH_3$	OH	H
Lactone from p-amino-2'-hydroxyglutethimide	Structure shown above		
p-Amino-1'-hydroxyglutethimide	NH_2	H	OH

5. METHODS OF ANALYSIS

5.1 Titrimetric Non-aqueous

Aminoglutethimide has been assayed in bulk and pharmaceutical formulations by non-aqueous titration using 0.1 N perchloric acid in glacial acetic acid (8). The end point is determined potentiometrically using a glass electrode and a calomel electrode as a reference electrode with a saturated solution of potassium chloride in methanol as the bridge fluid.

Agarwal and Blake(33) determined aminoglutethimide,

glutethimide and bemegride by dissolving the sample in (~1 milliequivalent of the powdered drug or tablets) in dimethylformamide (40 ml) using 0.1N sodium methoxide in benzene-methanol as a titrating agent. The end-point is determined potentiometrically (with calomel and platinum electrodes), or visually with azoviolet as indicator. The coefficient of variation is ~ 0.7%.

5.2 Spectrophotometric Analysis

Bult and Klasen(34) determined aminoglutethimide among other drugs colorimetrically through its reaction with cobaltous amine reagent (Parri reaction). The violet color produced could be measured at 555 and 530 nm, respectively. The general procedure is described as follows:

About 1 mg of the drug is dissolved in 1.6 ml ethanol. After addition of 0.2 ml cobaltous salt solution (cobaltous) nitrate 6 H_2O: 120 mg/20 ml methanol or cobaltous acetate 4 H_2O:100 mg/20 ml methanol) and 0.2 ml cyclohexylamine solution (6 g/20 ml methanol) the produced colour is compared with that of the blank (reagents).

Another method used to assay aminoglutethimide

colorimeterically in biological fluid (urine) is based on the formation of colored Schiff's base with p-dimethylaminobengaldehyde (Ehrlich's reagent). The yellow color formed was measured at 440 nm. A linear relationship between color intensity and concentration of the drug was obtained for the range of 1-15 μ g/ml (19). A modified method was applied to determine aminoglutethimide serum levels using Erlich's reagent (30). The authors reported that although several substances could potentially interfere with the blood assay of aminoglutethimide, none was actually found to do so to any significant degree.

5.3 Chromatography

5.3.1 Paper Chromatography

Douglas and Nicholls (19) described a method for identification and separation of aminoglutethimide in urine of man by paper chromatography. The chromatograms run on Whatman No. 1 paper by ascending technique using solvent systems shown in Table 3.

Table 3. Solvents Systems used for Identification of Aminoglutethimide by Paper Chromatography

	Solvent System	R_f	Localizing Agent
a)	n-BuOH:HOAc:H_2O 12: 3:5 v/v	0.73	a) N,N-dimethylaminocinnamldehyde spray (permanent red color) (35)

b) $CHCl_3$: MeOH 1:1 v/v	0.93	b) UV light
c) CCl_4: HOAc: H_2O 1: 2: 1 v/v	0.34	c) Erlich's reagent (yellow (color)
d) Aqueous NaCl 10% w/v	0.74	d) Nitrous acid-naphthylethylene diamine(35)
		e) NaOCl-KI-starch (blue black color)(36)

Davies and Nicholls(38) reported a study of the chromatographic behavior of nine glutarimides including aminoglutethimide using Whatman No. 1 paper impregnated with liquid paraffin (4% in hexane), olive oil (20% in acetone) or tributyrin (10% in acetone). The most useful solvent system used were:

a) toluene: acetic acid: water 10:5:4

b) carbon tetrachloride: acetic acid: water 1:2:1

c) 10% w/v aqueous sodium chloride

d) 0.066M sodium phosphate (pH7.3)

The compounds were detected by hypochlorite reagent or alkaline hydroxylamine reagent.

5.3.2 Thin Layer Chromatography (TLC)

Several procedures had been published for detection of aminoglutethimide by tlc which is summarized in Table 4.

Table 4. Thin Layer Chromatography of Aminoglutethimide

Solvent System	Visualizing Agent	Comments/Reference
$CHCl_3:C_6H_6$ 1:1	Hydroxylamine reagent	Alumina 50 μg thick plate activated at 150° for 1 hour was used (37)
$C_6H_5CH_3:CH_3COCH_3$ 90:60	Ehrlich's reagent	used in determination of aminoglutethimide in serum (30)
iso $PrOH:CHCl_3:25\%NH_4OH$ 9:9:2 $CHCl_3:CH_3COCH_3$	Dichlorofluoroscein or Mercuric nitrate	- (38)
$CHCl_3:CH_3COO_2CH_5$ 35:15	UV light at 254 nm	used in identification of aminoglutethimide in bulk (R_f:0.4) and to detect the by-product m-aminoglutethimide (R_f:0.35) MNSil G25 HR/UV plate (Macherey-Nagel & Co. was 8 used. (8)
$CH_3COOC_2H_5:CH_3OH:$ 100%CH_3COOH 17:3:0.1	UV light at 254 nm or by diazotization and spraying with napthyl ethylenediamine dihychloride.	applied to identify aminoglutethimide in tablets (R_f:0.6) Silica gel 60F 254 plate used. (8)

5.3.3 Gas-Liquid Chromatography (GLC)

Adams and Roger(39) recently published a rapid, sensitive and selective GLC assay for aminoglutethimide in biological fluids. The method is suitable for the study of the pharmacokinetics of the drug and its N-acetyl metabolite (which gave an assymmetric peak shape on this system). The conditions are as follows:

Column: glass column (1.5in x 0.4mm I.D.) packed with 2% CDMS (cyclohexanedimethanol succinate) on Chromosorb W80-100 mesh (acid washed & dichloromethylsalisilane treated).

W80-100 mesh (acid washed & dichloromethylsalisilane treated).

Column temperature	:	240°
Detector temperature	:	350°
Carrier gas	:	Nitrogen
Flow rate	:	100ml/min
Detector	:	Nitrogen selective

Dutt reported a gas chromatographic method for the identification of 116 common drugs by their multiple peaks of the parent and their

trimethylsilylyl derivatves including aminoglutethimide (40). The study was done using 3% OV-17 Gas-Chrom Q 100-120 mesh glass column (2m x 3mm I.D.) and flame ionization detector (FID). The carrier gas was nitrogen at a flow rate of 30 ml/min and the hydrogen and air flow rates were 30 and 450ml/min, respectively. The injection port and detector temperatures were 300° and the oven temperature was programmed from 120° to 270° at 10°/min. or at an isothermal temperature of 300°.

5.3.4 High-Performance Liquid Chromatography (HPLC)

Several HPLC procedures have been published for the determination of aminoglutethimide and its major metobolite (N-acetylaminoglutethimide) in biological fluids. Table 5 summarises some of the HPLC systems used for the analysis of the drug.

Table 5 HPLC Systems Aminoglutethimide

Column	Mobile Phase	Detection	Comment/Reference
Spherisorb ODS column (30cm X 4mm I.D., 5μg particle size	MeCN: 0.01 M phosphate buffer pH 6.8 (22:68), flow rate 1.5 ml/min	UV at 234 nm	Applied for simple determination of aminoglutethimide & its metabolite (41)
Octadecyl (C18) silica reversed phase uncapped column (10cm X 0.8cm I.D. with 10μm particle size).	MeCN: tert. butylammonium phosphate:H_2O (100:3:100), adjusted to pH 6.3±0.2 with orthophosphoric acid, flow rate 2 ml/min.	UV at 254 nm	Applicable for assay of the drug in plasma (42)
ODS Hypersil (10 cm long, 3μm particle size	11% MeCN in 100mM ammonium phosphate buffer, pH 3.5 also - 8-20% MeCN in 15mM acetate buffer pH 4.5 - or 10mM phosphate buffer, pH 6.0, flow rate 2 ml/min. Elution of nitroglutethimide required a step wise increase in MeCN conc. from 11 to 23% after 16 min.		Applicable for analysis of the drug and its metabolites in plasma. Retention times were approx.: 4.5 min aminoglutethimide, 14 min. N formylmetabolite, 17 min N-acetylmetabolite, 28 min. nitroglutethimide (43).

Table 5 HPLC Systems Aminoglutethimide

Column	Mobile Phase	Detection	Comment/Reference
Nucleosil - 10 (C18) stainless steel column (20 cm x 4.8 mm I.D.)	MeCN:H_2O:Et_3N (25:75:0.05) flow rate 2 ml/min	UV at 254 mm	Applied as stability indicating assay (8)
Nucleosil - C18 (5µm particle size)	MeCN: MeOH:H_2O (5:20:75)	UV at 235 mm	Applied for simultaneous determination of the drug and its major metabolite in human plasma (44,45) Retention times for aminoglutethimide = 8.9 min N-acetylmetabolite = 12.1 min
Reversed phase Magnusphere C18 (7µm particle size 15 cm x 4.6 cm I.D.) stainless steel column	Citrate buffer pH 3-4: MeOH (500: 280), flow rate 1.2 ml/min	UV at 254 mm	Suitable for pharmacokinetic study of aminoglutethimide and its N-acetyl metabolite in biological fluids (plasma, urine saliva), (39)

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CAFFEINE

Mohammad Uppal Zubair

Mahmoud M.A. Hassan, and

Ibrahim A. Al-Meshal

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

a) 1H-Purine-2,6-dione, 3,7-dihydro-1,3,7-trimethyl.

b) 1,3,7-Trimethylxanthine.

c) Methyltheobromine.

d) 1,3,7-Trimethyl-2,6-dioxo-1,2,3,6-tetrahydropurine.

e) 3,7-Dihydro-1,3,7-trimethyl-1H-purine-2,6-dione.

f) 1,3,7-Trimethyl-4,6-dioxopurine.

1.1.2 Generic Names

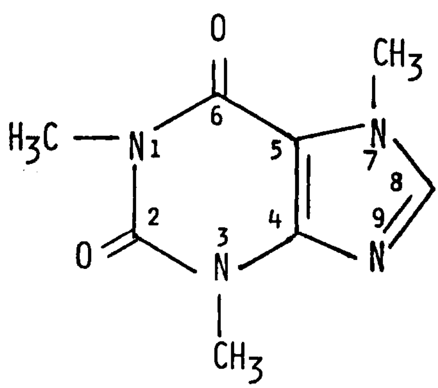
Anhydrous caffeine; Caffeine; Coffenium; Coffeine; Coffeina; Guavanine; Methyltheobromine; No-Doz; Thiene.

1.2 Formulae

1.2.1 Empirical

$C_8H_{10}N_4O_2$.

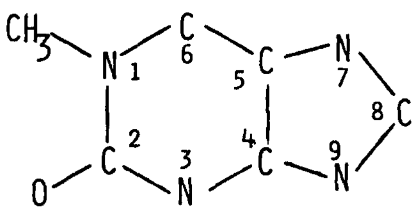
1.2.2 Structural



Discovered by Robiquet in coffee in 1821, while searching for quinine which he believed to be present. In 1827, Oudry found an alkaloid in tea and called it thiene. In 1838, Jobst and Mulder proved the identical character of the two principles (1).

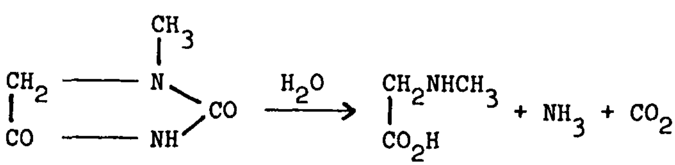
1.2.3 Structural Confirmation by Degradation (2,3)

Fischer has reported that caffeine on oxidation gave dimethylalloxane and methyl urea, indicating similarity with the skeleton structure of uric acid.



With the above information it becomes evident that : the third methyl group is either at position 7 or 9 and the remaining oxygen atom is either at position 6 or 8.

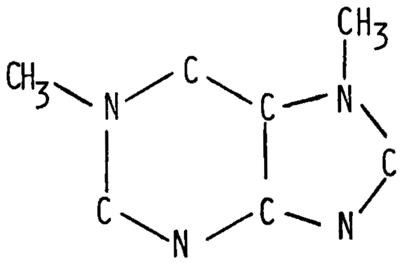
Position of the methyl group; Fischer also isolated another oxidation product which on hydrolysis, gave N-methylglycine, carbondioxide and ammonia. Thus, this third oxidation product must be N-methylhydantoin.



N-methylhydantoin

Therefore, it follows that caffeine contains two ring structures that of dimethylalloxane and that of methylhydantoin. The following two skeleton

structures were proposed:



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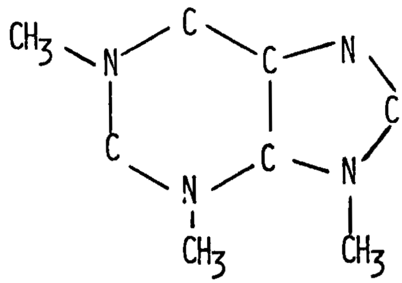
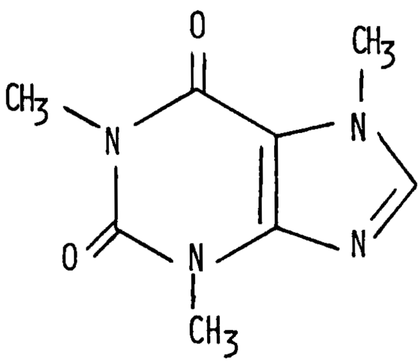


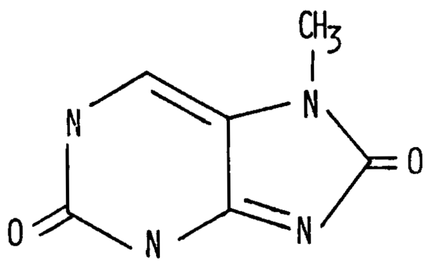
II

Finally Fischer isolated fourth oxidation product, N,N'-dimethyloxamide $CH_3NHCOCONHCH_3$. Examination of (I) and (II) showed that only (I), could give rise to the formation of the oxamide. Therefore (I) must be the skeleton of caffeine.

Position of oxygen atoms: the following two structures were possible with regard to the position of oxygen atoms.



III



IV

By analogy, it would appear that the more likely structure of III would be that of uric acid.

Fischer, further confirmed the caffeine structure with the following degradation studies:

$$\text{Caffeine} \xrightarrow{Cl_2} \text{Chlorocaffeine} \xrightarrow[\text{NaOH}]{CH_3OH} \text{Methoxycaffeine}$$

$$\text{Methoxycaffeine} \xrightarrow{\text{dilute HCl, boil}} \text{Oxycaffeine} + CH_3Cl$$

1.2.4 <u>CAS Registry Number</u>

a) Anhydrous caffeine [58-08-2]
b) Monohydrate [5743-12-4]

1.2.5 <u>Wiswesser Line Notation</u>

T 56 BN DN FN
VNVJ B F H

1.3 <u>Molecular Weight</u>

Anhydrous $C_8H_{10}N_4O_2$ = 194.19

Monohydrate $C_8H_{10}N_4O_2 \cdot H_2O$ = 212.21

1.4 <u>Elemental Composition</u>

C, 49.48% ; H, 5.19% ; N, 28.85% ; O, 16.48%.

1.5 <u>Appearance, Color, Odor and Taste</u>

White powder or white, glistening needles, usually matted together. It is odorless and has a bitter taste.

2. <u>Physical Properties</u>

2.1 <u>Melting range</u>

Between 235° and 237.5°, determined after drying at 80° for 4 hours.

Hexagonal prisms by sublimation, m.p. 238° (4)

2.2 <u>Sublimation</u>

Caffeine sublimes at 178°. Fast sublimation is obtained at 160-165° under 1 mm pressure at 5 mm distance.

2.3 Density

d_4^{18} 1.23 (5).

2.4 Solubility (1)

1 gm of anhydrous caffeine dissolves in about 50 ml water, 6 ml water at 80°, 75 ml alcohol, about 25 ml alcohol at 60°, about 6 ml chloroform, 600 ml ether, 50 ml acetone, 100 ml benzene and 22 ml boiling benzene. Freely soluble in pyrrole; in tetrahydrofuran containing, 4% water, also soluble in ethylacetate and slightly in petroleum ether.

Being a weak base, caffeine does not form stable salts, and even its salts of strong acids, such as the hydrochloride or hydrobromide, are readily hydrolysed by water. The solubility of caffeine in water is increased by the presence of organic acids or their alkali salts, e.g., benzoates, salicylates, cinnamates, or citrates and this is the reason for the use of several such preparations.

2.5 Crystal Structure

Sutor (6) has determined the crystal structure of caffeine, 1,3,7-trimethyl-2,6-dihydroxy purine (Fig. 1). Crystallographically, it is nearly isomorphous with theophylline (Fig. 2) (7), and a comparison of bond lengths in the two has yielded information concerning the effects of a substituent in the imidazole ring on the purine ring. The crystals of caffeine are monoclinic, space group $P2_1/a$, with a = 14.8 ± 0.01, b = 16.7 ± 0.1, C = 3.97 ± 0.03 A°; β = 97.0 ± 0.5°.

Systematic absences are hoc with h = 2n + 1, OKO with K = 2n + 1; space group $P2_1/a$ -C^5_{2h}. Its final Fourier projection is shown in Fig. 3. Table 1 shows the fractional coordinates referred to the monoclinic crystal axes. The crystal structure was solved by an application of the isomorphus-replacement method and a consideration of the possible hydrogen bond system in the crystal. Bond lengths and bond angles within the molecule are shown in Fig. 4, Fig. 5 and Table 2.

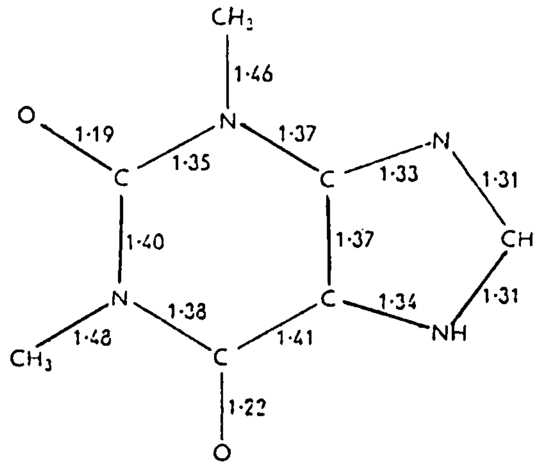


Fig. 2: Crystal structure of theophylline.

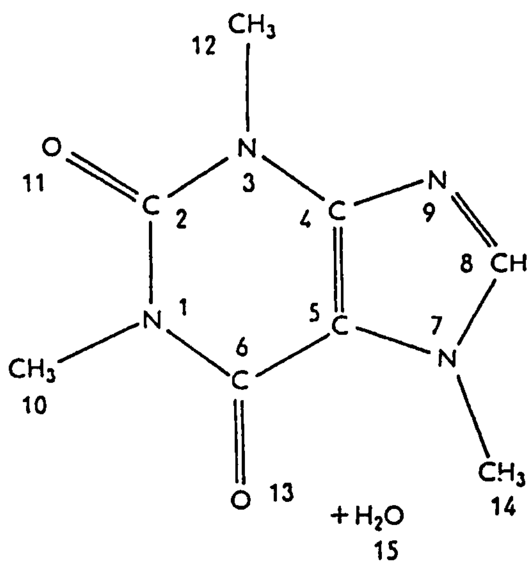


Fig. 1: Caffeine, showing the numbering system used.

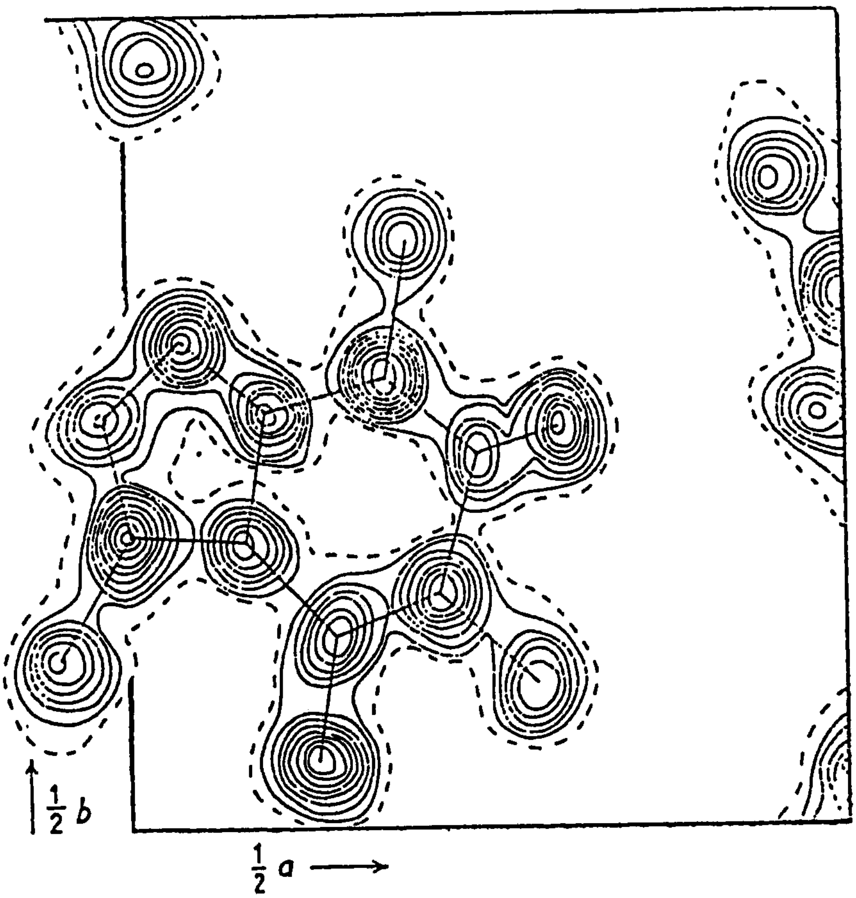


Fig. 3: The final UKO Fourier projection of caffeine, contours are arbitrary but equal intervals; ZUO contour broken.

Table 1. The Fractional Coordinates Referred to the Monoclinic Crystal Axes.

	x	y	z	x*	y*
C_2	0.2416	0.2225	0.9002	0.2414	0.2225
C_4	0.1003	0.2533	0.1295	0.1019	0.2541
C_5	0.0835	0.1769	0.1944	0.0841	0.1759
C_6	0.1456	0.1151	0.1155	0.1463	0.1143
C_8	0.4798	0.2481	0.3638	0.4801	0.2480
C_{10}	0.2879	0.0841	0.8790	0.2891	0.0832
C_{12}	0.1960	0.3641	0.9209	0.1959	0.3638
C_{14}	0.4533	0.3950	0.4584	0.4536	0.3947
N_1	0.2193	0.1418	0.9735	0.2196	0.1415
N_3	0.1796	0.2764	0.9848	0.1801	0.2769
N_7	0.0009	0.1749	0.3376	0.0020	0.1749
N_9	0.0407	0.3008	0.2440	0.0403	0.3008
O_{11}	0.3057	0.2397	0.7614	0.3063	0.2400
O_{13}	0.1374	0.0404	0.1616	0.1363	0.0404
O_{15}	0.0166	0.4790	0.2705	0.0184	0.4705
H_1	0.413	0.239	0.474	-	-
H_2	0.487	0.438	0.599	-	-
H_3	0.435	0.437	0.278	-	-
H_4	0.395	0.363	0.510	-	-
H_5	0.263	0.362	0.857	-	-
H_6	0.228	0.396	0.105	-	-
H_7	0.142	0.377	0.783	-	-
H_8	0.348	0.100	0.772	-	-
H_9	0.300	0.033	0.022	-	-
H_{10}	0.257	0.060	0.676	-	-

x* and y* represent the weighted x and y coordinates from the hKO and hKL projections.

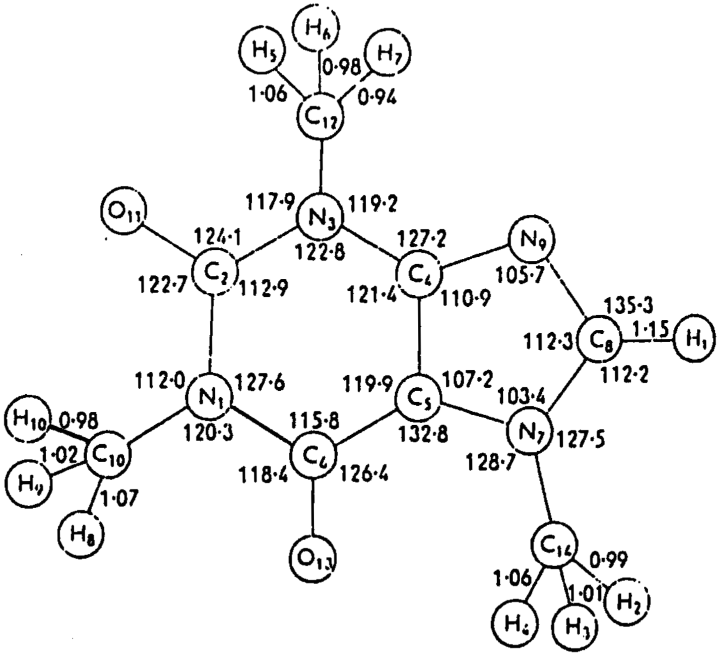


Fig. 4: Bond angles and C-H bond lengths in the caffeine molecule.

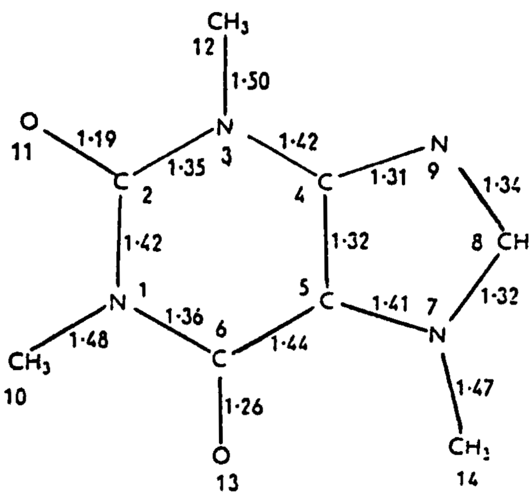


Fig. 5: Bond lengths in the caffeine molecules.

Table 2. Bond Lengths and their Standard Deviation

Bond	Bond length (Å)	Standard deviation (Å)
C_2-N_1	1.42	0.014
C_2-N_3	1.35	0.016
C_4-N_3	1.42	0.021
C_4-C_5	1.32	0.014
C_5-C_6	1.44	0.015
C_6-N_1	1.36	0.021
C_4-N_9	1.31	0.019
C_8-N_9	1.34	0.019
C_8-N_7	1.32	0.012
C_5-N_7	1.41	0.021
C_{10}-N_1	1.48	0.016
C_{12}-N_3	1.50	0.013
C_{14}-N_7	1.47	0.018
C_2-O_{11}	1.19	0.023
C_6-O_{13}	1.26	0.013

A comparison is made of the intramolecular distances with those in other purines and a pyrimidine, Table 3, and indicates that steric hindrance must be allowed for, in a theoretical calculation of bond length in relatively complicated molecule of this type. Evidence for and against the existence of a short hydrogen bond between water molecules is given.

Crystal structure of 2:1 molecular complexes of caffeine with hexaaquamagnesium (II) bromide and hexaaquamanganese (11) Triiodide iodide crystal structures of

Table 3. Significantly Different Bond Lengths in a Comparison of Caffeine with Theophylline, Adenine Hydrochloride and Uracil.

Caffeine with theophylline

Bond	Significant difference (A°)	Actural difference (A°)
C_4-C_5	0.04	0.05
C_5-N_7	0.05	0.07

Caffeine with uracil

Bond	Significant difference (A°)	Actual difference (A°)
C_4-N_3	0.06	0.08
C_4-C_5	0.05	0.09

Caffeine with adenine hydrochloride

Bond	Significant difference (A°)	Actual difference (A°)
C_2-N_1	0.05	0.05
C_2-N_3	0.05	0.05
C_4-N_3	0.06	0.06
C_4-C_5	0.05	0.05
C_4-N_9	0.05	0.05

$(C_8H_{10}N_4O_2)_2Mg$-$(OH_2)_6Br_2$ and $(C_8H_{10}N_4O_2)_2Mn(OH_2)_6I.I_3$ have been determined by X-ray diffraction methods (8).

The crystal structures of $(C_8H_{10}N_4O_2)_2Mg(OH_2)_6Br_2$ (I) and $(C_8H_{10}N_4O_2)_2Mn(OH_2)_6I.I_3$ (II) have been determined by X-ray diffraction methods; crystals of I are triclinic, space group P1, with Z = 1, in a unit cell of dimension: a = 9.620(7), b = 10.779(8), c = 7.645(6) A°, α = 107.03(7), β = 108.88(7), γ 72.71(8)°; crystals of

II are monoclinic, space group $P2_1/n$, with Z = 4, in a unit cell of dimensions a = 12.406(8), b = 29.652(12), c = 9.419(6) A°, β = 108.39(7)°. The structures of I and II have been solved from diffractometer data by Patterson and Fourier methods and refined by fullmatrix least-squares to R = 0.046 for I and 0.090 for II. Both compounds contain octahedral hexaaquametal (II) cations, uncoordinated caffeine molecules and bromide anions in I and triiodide and iodide anions in II, held together by a network of hydrogen bonds. The triiodide anions are unsymmetrical [I(1)-I(2) = 2.89, I(2)-I(3) = 2.95 A°, I(1)-I(2)-I(3) = 178°], arranged in linear systems with a weak 'head-to-tail' interaction, the distance being of 3.62 A°.

2.6 Spectral Properties

2.6.1 Ultraviolet Spectrum

The UV spectrum of caffeine in methanol (Fig. 6) was scanned from 190 to 440 nm, using DMS 90 Varian Spectrophotometer. It exhibited a λ_{max} at 270 nm. Other reported UV spectral data are shown below:

Solvent	λ_{max} nm	E1%, 1 cm	Ref.
Methanol	272	-	9
Trichloro-ethylene	278	-	10
Ethanol	273	519	11
0.1N HCl	272	470	11
0.1N HCl	275	490	12
0.1N NaOH	275	490	12

2.6.2 Infrared Spectrum

The IR spectrum of caffeine as KBr disc (Fig. 7) was recorded on a Perkin Elmer - 580 B Infrared spectrophotometer to which an Infrared data station is attached. The structural assignments

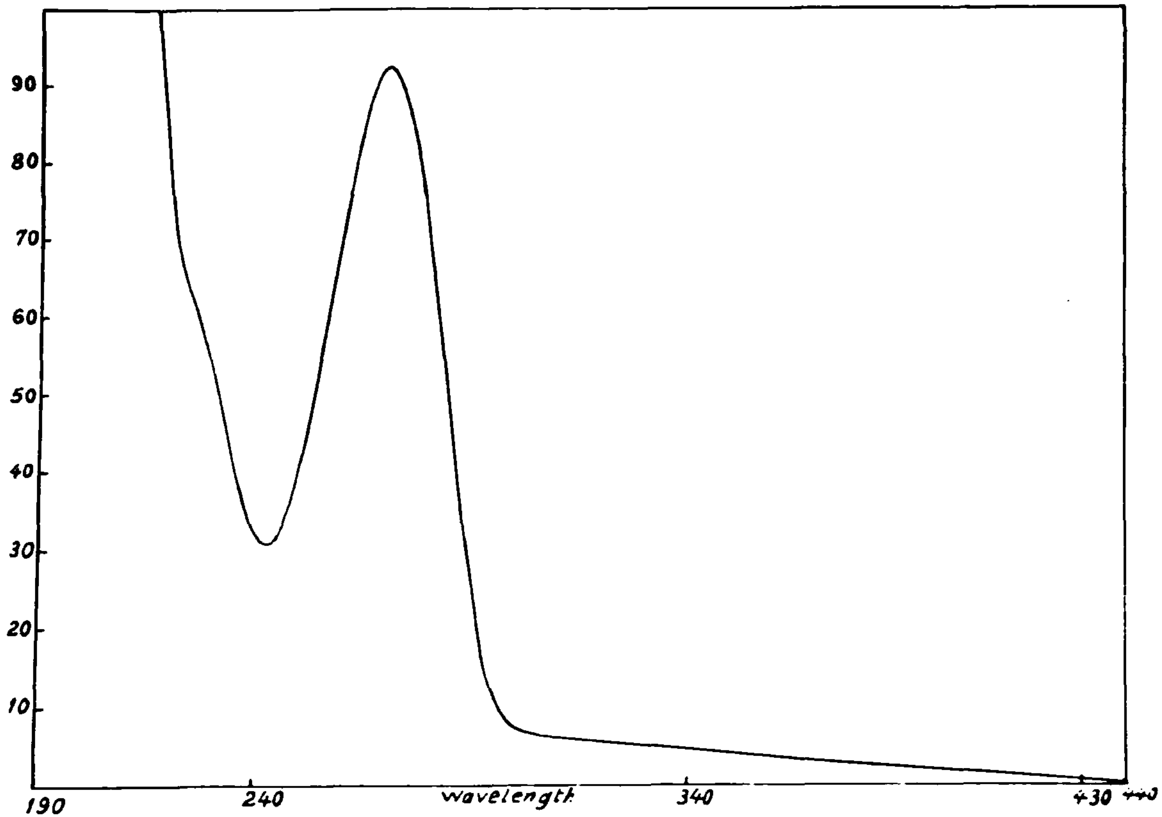


Fig. 6 UV Spectrum of Caffeine in Methanol.

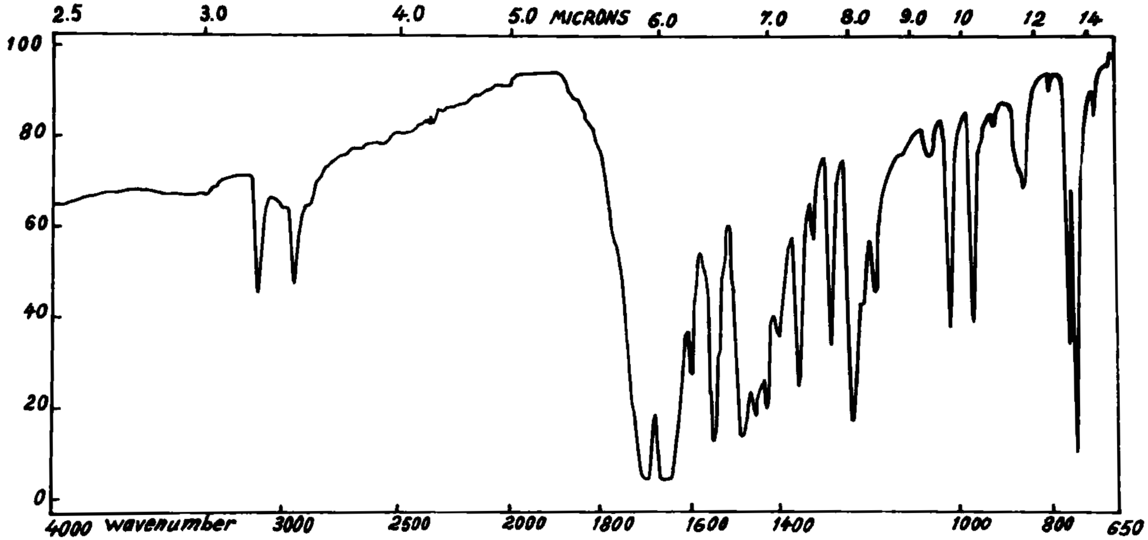


Fig.7 IR Spectrum of Caffeine as KB disc

have been correlated with the following frequencies Table 4).

Table 4. IR Characteristics of Caffeine.

Frequency cm^{-1}	Assignment
3110, 2950	CH_3 and CH stretch
1700	C = O
1650	C = N

Other characteristic bands are:
1600, 1550, 1480, 1455, 1430, 1400, 1360, 1285, 1235, 1185, 1020, 970, 860, 760 and 745.

The infrared data for caffeine were also reported and are as follows:
3100, 2970, 1700, 1660, 1550, 1480, 1360, 1240, 1020, 980, 750, and 610 cm^{-1} (9).

Principal peaks are 1658, 1695 and 745 cm^{-1} (11). Major peaks 747, 1454, 1480, 1548, 1658 and 1698 cm^{-1} (12). The region from 19-22 μ is important for identification of caffeine (13).

2.6.3 Nuclear Magnetic Resonance Spectra

2.6.3.1 Proton Spectrum

The PMR spectrum of caffeine in $CDCl_3$ (Fig. 8) was recorded on a Varian T60-A, 60 MHz NMR spectrometer using TMS (tetramethylsilane) as an internal reference. The following structural assignments have been made (Table 5).

Table 5. PMR Characteristic of Caffeine

Group	Chemical Shift (δ) ppm
1-N-CH_3	3.53 s
3-N-CH_3	3.33 s
7-N-CH_3	3.98 s
8-H	7.54 s

s = singlet

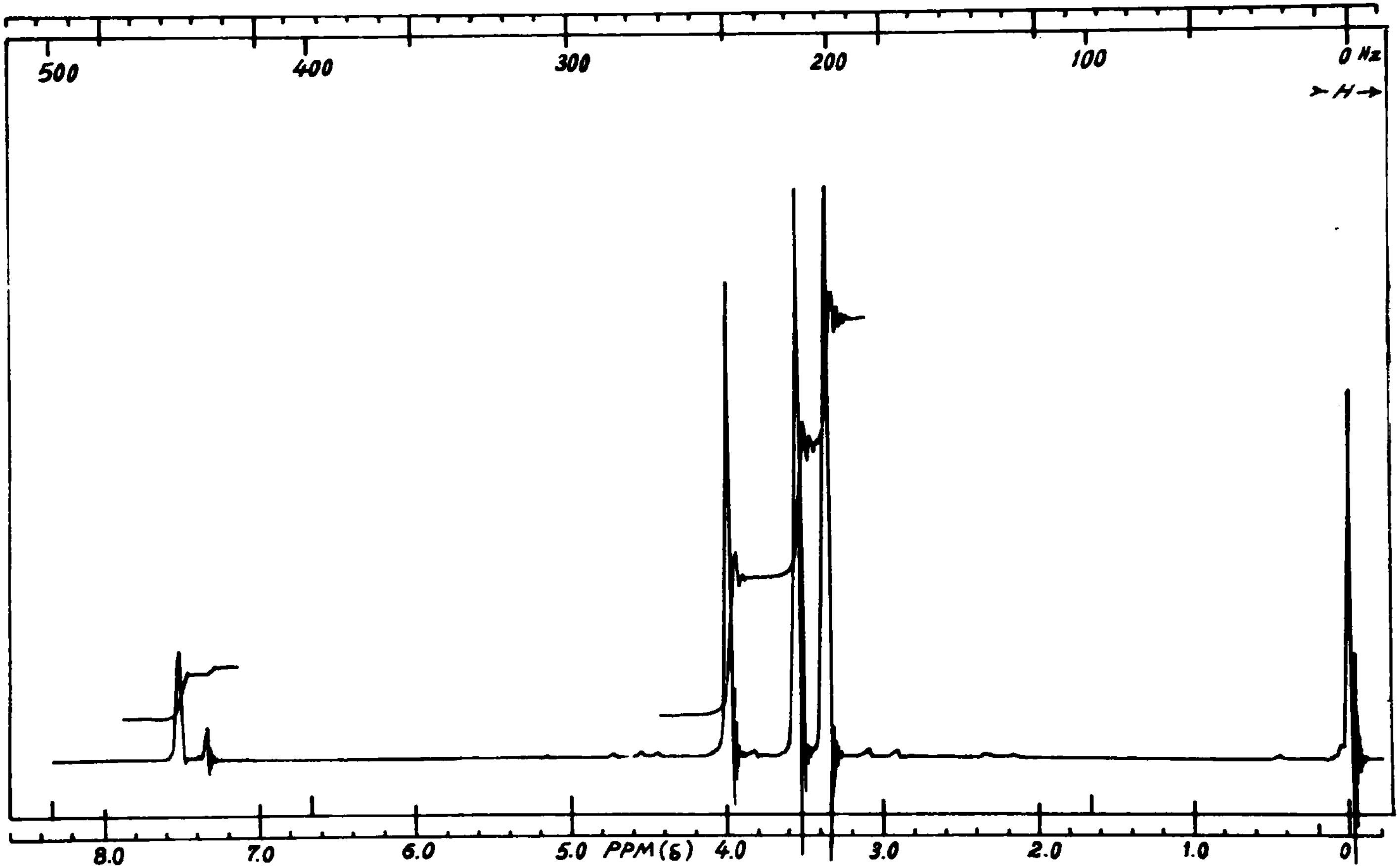
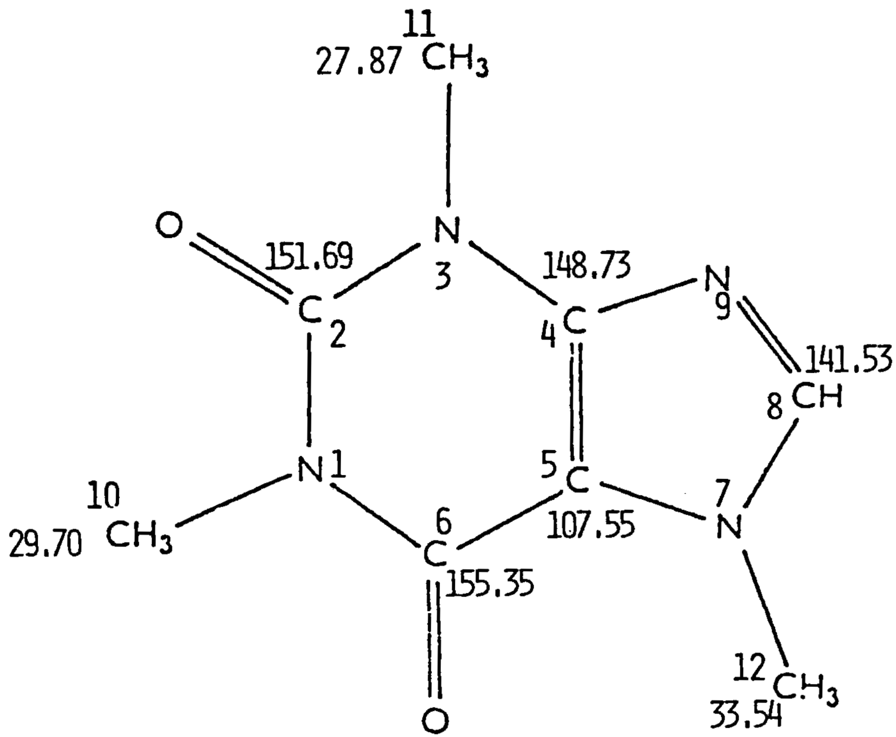


Fig.8 PMR Spectrum of Caffeine in $CDCl_3$



The structure of caffeine for carbon chemical shifts.

Other reported data are 3.4, 3.6, 4.0 and 7.6 ppm (9,14). Stamm (15) has investigated the association of caffeine with sodium benzoate and other compounds and reported a large chemical shift of the NMR signals of the three methyl groups. Also metal porphyrin/caffeine complexes have been investigated (16).

2.6.3.2 ^{13}C-NMR Spectra

The natural abundance C-13 NMR noise decoupled and single frequency off-resonance decoupled (SFORD) spectra (Figs. 9 and 10) were obtained at 20 MHz on a Varian FT-80A, 80 MHz Fourier transform NMR spectrometer using a broad band 10 mm probe. The sample was run at concentration Ca 1-2 M in deutrated chloroform with tetramethylsilane as an internal reference standard. The chemical shifts were measured at 5 KHz spectral width. The carbon chemical shifts are assigned on the basis of the theory of chemical shift and SFORD splitting pattern and is shown in Table 6.

Table 6. Carbon Chemical Shifts of Caffeine.

Carbon No.	Chemical Shift (δ) ppm	Multiplicity
C-2	151.69	s
C-4	148.73	s
C-5	107.55	s
C-6	155.35	s
C-8	141.53	d
C-10-CH_3	29.70	q
C-11-CH_3	27.87	q
C-12-CH_3	33.54	q

s = singlet; d = doublet; q = quartet.

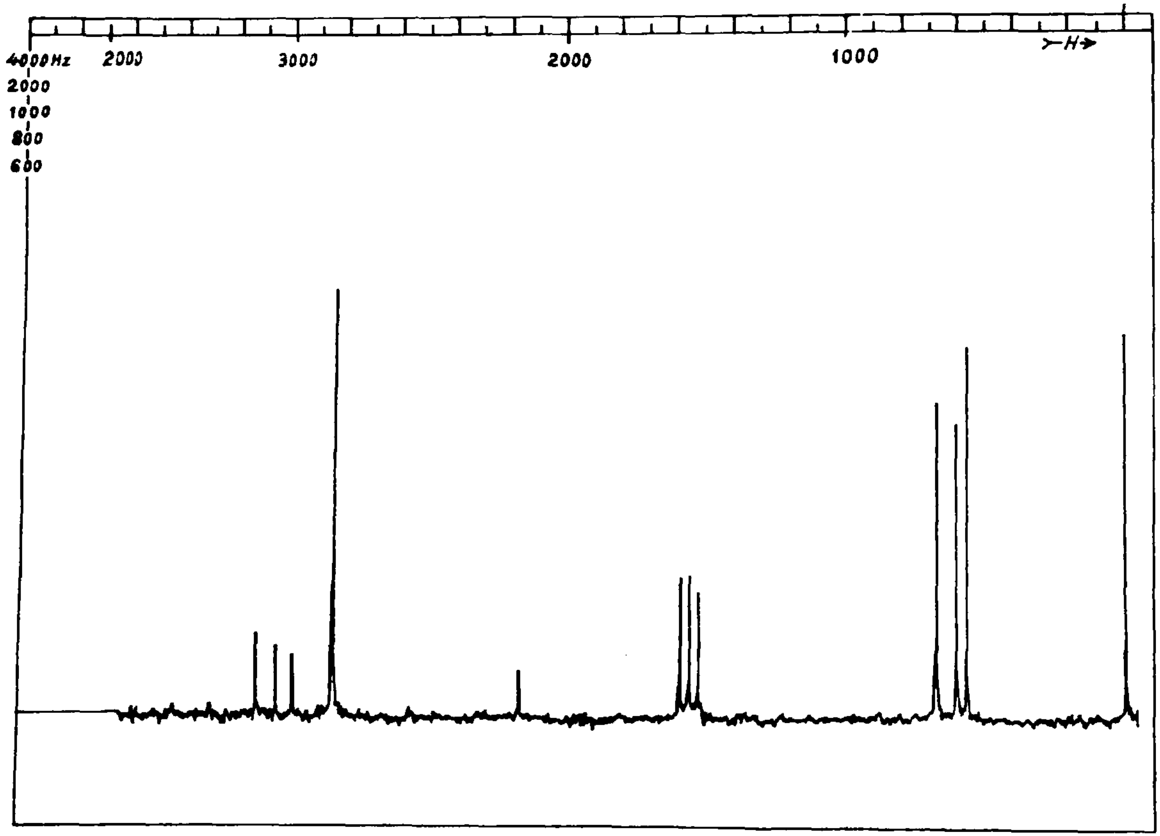


Fig. 9 ^{13}C-NMR Noise-decoupled Spectrum of Caffeine in $CDCl_3$

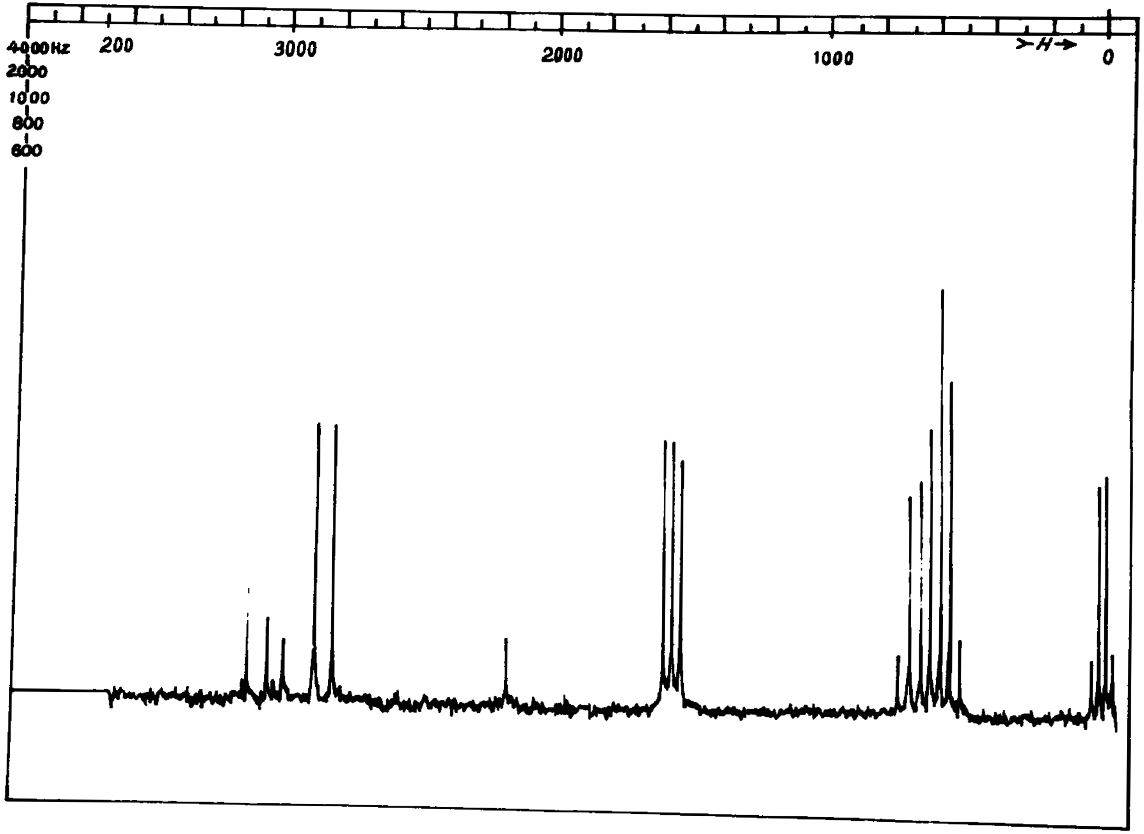


Fig. 10 ^{13}C-NNR off Resonance Spectrum of Caffeine in $CDCl_3$

2.6.4 Mass Spectrum

The mass spectrum of caffeine obtained by electron impact ionization (EI) is shown in Fig. 11. It was recorded on Finigan-Mat 1020 GC/Mass spectrometer. The spectrum was scanned from 40 upto 440 a.m.a. Electron energy was 70 eV. The mass spectral data are shown in Table 7.

Table 7. The Most Prominent Fragments of Caffeine.

m/e	Relative intensity %	Fragment
194	100	Base peak (M^+)
109	66	$C_5H_7N_3$
82	37	-
67	54	-
55	80	-

Other mass spectral data were also reported (9,17) 194[100], 67[66], 109[66], 82[39], 42[28], 40[18] and 41[16]. A comparison of mass spectral data of theobromine, theophylline and caffeine was reported by Spiteller and Friedmann (18).

Dunbor and Wilson have published an isotope mass spectrum method for identification of the geographical origin of caffeine (19).

3. Isolation of Caffeine

3.1 From Tea Leaves : Finely powdered tea leaves (100 g) are extracted with ethanol in a soxhlet apparatus for 3 hrs (20). The caffeine so extracted is then adsorbed on magnesium oxide. It is then desorbed after treatment with 10% H_2SO_4 and is extracted into chloroform and is recrystallised.

3.2 From Green Coffee : A procedure for the extraction of caffeine from green coffee has been reported (21). Green coffee beans were steamed for 2 to 3 hours to equilibrate the water content between 40 and 50%.

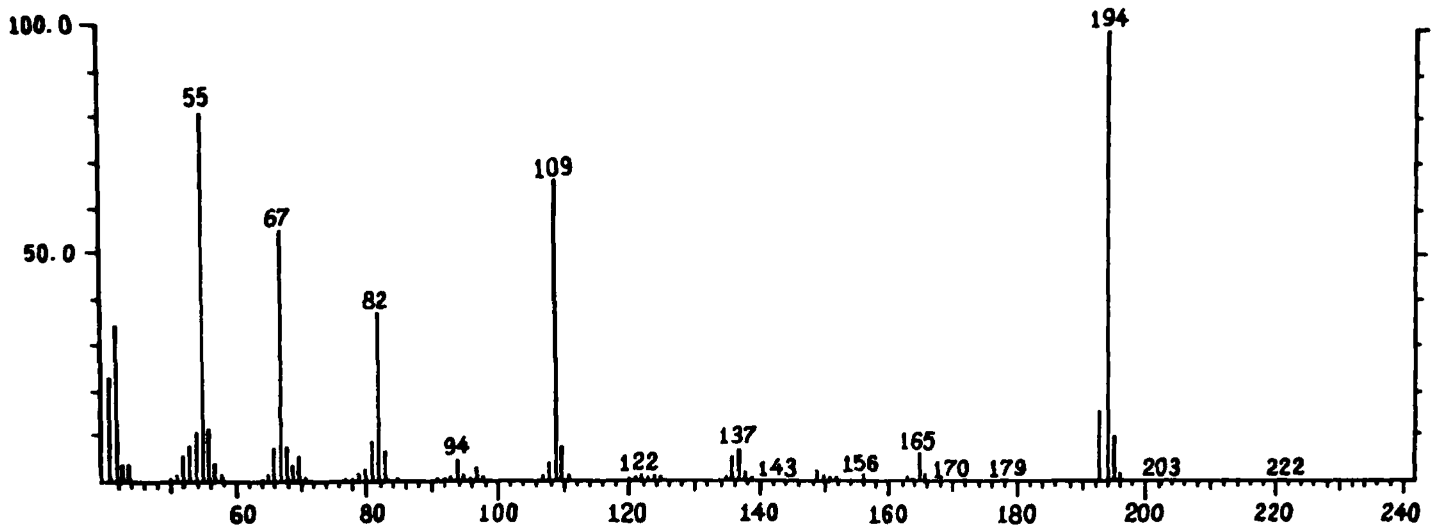


Fig. 11. EI-Mass Spectrum of Caffeine.

Caffeine was then extracted with $CHCl_3$ and caffeine upto 95% of the theoretical amount was recovered.

3.3 A process for the extraction of caffeine from coffee beans by leaching with water has been developed (22). The highest yield of 90.4% was obtained when the average coffee particle size was 1.4095 mm, the water/coffee ratio of 9:1, at 75°C and the extraction was carried out for thirty minutes.

3.4 Natural caffeine is also obtained as a by-product, from the decaffeination process of coffee and tea. Some of these method are summarised and presented in Table 8.

3.5 Caffeine can also be obtained from various sources. Table 9 summarises the source, botanical characteristics, occurrance and the caffeine/theobromine content in each case (37).

Table 8. Isolation of Caffeine by Decaffeination Processes.

Material	Solvent	Ref.
Green coffee beans*	Fluorinated hydrocarbons	23
Coffee* and tea extracts (also oil caffeine).	With oils such as corn oil, olive oil, safflower oil.	24
Coffee base*	Benzyl alcohol	25
Instant coffee	-	26
Black tea*	With supercrit carbon dioxide.	27
Tea waste	-	28
Green coffee*	Alcohols	29
Caffeine*	Supercrit carbon dioxide with water.	30
Coffee and tea	With supercrit carbon dioxide.	31
Roasted coffee* (aqueous extracts of)	With supercrit carbon dioxide and recovery of aroma.	32
Raw coffee*	Supercrit carbon dioxide.	33
Coffee*	Propane and butane	34
Coffee*	Aqueous (solutions)	35
Coffee*	Aqueous (steam)	36

Table 9. Plant Sources of Caffeine

Source	Caffeine (theobromine) content (%)	Information about the plant		
		Botanical characteristics	Native region	Main region of cultivation
Seeds (beans) of *Theobroma cacao* (sterculi-aceae).	0.1-0.8 (0.2-2.7)	Tree, 4-15 m in height when growing wild, but pruned to 6 m under cultivation. Fruits furrowed leathery pods, shaped like large cucumbers and containing 20-25 cream coloured almond-shaped seeds.	Mexico, Central and South America	West Africa
Seeds (beans) of *Coffea arabica*, *C. liberica*, *C. excelsa* or *C. robusta* (Rubiaceae)	0.8-2.4	*C. arabica:* Shrub or small tree, 4-6 m in height when growing wild but pruned to 2-3 m under cultivation. Fruits ellipsoidal berries (cherries), red to dark purplish, containing 2 silvery skinned seeds (Beans) within the pulp.	East Africa	South America

Continued Table 9.

Source	Caffeine (theobromine) content (%)	Information about the plant		
		Botanical characteristics	Native region	Main region of cultivation
Seeds (beans) of *Paullinia cupana* (Sapindaceae)	2-6	Large, climbing or creeping plant with smooth stem. Fruit ovoid, nut-like, about as large as a grape and usually containing 1 seed, the size of a hazel-nut, with white, mealy covering.	Amazon valley of Brazil	State of Amazons, Brazil.
Bark of *Paullinia yoco* (Sapindaceae)	2.7	Extensive, woody liana. Stem stout, up to 12 cm in diameter at the base, with a milky-white astringent sap.	Colombia, Equador, Peru	Not cultivated.
Leaves of *Ilex paraguayensis* (Aquifoliaceae)	1.1-1.9	Tree, 20-30 m in height when growing wild, 4-6 m under cultivation. Leaves Persistent alternate, finely toothed at the margin and dark green in colour.	Large area in valleys of the Parana, Paraguay and Upper Uruguay rivers.	Paraguay and South of Brazil

Continued Table 9.

Source	Caffeine (theobromine) content (%)	Information about the plant		
		Botanical characteristics	Native region	Main region of cultivation
Leaves and shoots of *Ilex vomitoria* (Aquifoliaceae)	0.1-1.6	Shrub or small tree, closely related to *I. paraguayensis*	East coast of North America from Virginia to Mexico.	Not cultivated.
Leaves of *Camellia sinensis* (Theaceae)	3-4	Shrub or small tree, 9-15 m in height when growing wild, about 1.5 m under cultivation. Leaves alternate, elliptical on short stalks, leathery and with toothed margins.	Assam, China, Japan.	Sri Lanka, India, China Japan.
Seeds (nuts) of *Cola nitida* and *C. acuminata* (Sterculiaceae). Most of the caffeine in kolas not derived from the kola nut but added in the form of synthetic caffeine.	1.5-3.5	*C. acuminata:* Slender tree, 6-9 m tall, trunk commonly branching near the base. Bark rough and corky, grey in colour. Foliage sparse, confined to the tips of branches. Fruit consists of up to five follicles borne at right angles to the stalk or slightly downwards. Follicles, russet, rough to the touch. Each follicle contains up to 14 pink or red seeds, covered with white skin. Cotyledons 3-6.	Southern Nigeria	West Africa

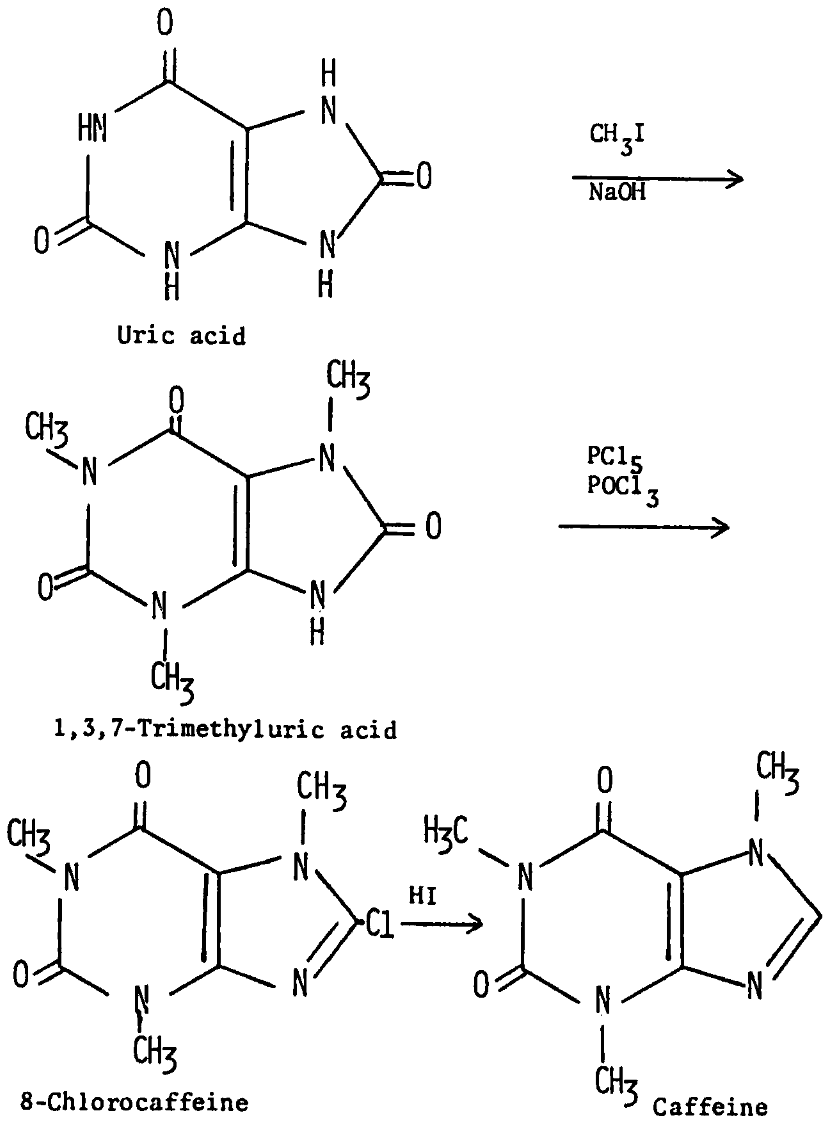
Continued Table 9.

Source	Caffeine (theobromine) content (%)	Information about the plant: Botanical characteristics	Native region	Main region of cultivation
In Africa, the seeds are used mainly as a masticatory, but they may also be used for preparation of a drink (Cola).		*C. nitida:* More robust tree, 9-12 m height, trunk unbranched for at least 1 m. Bark smooth with fine longitudinal cracks. Foliage dense, not confined to the tips of the branches. Fruit consists of up to five follicles, usually bent upwards. Follicles green, smooth to the touch. Seeds up to 10 in number, with 2 cotyledons and of the shape and size of horse chestnuts.	Sierra Leone, Ivory coast, Ghana	West Africa, West Indies South America

4. Synthesis

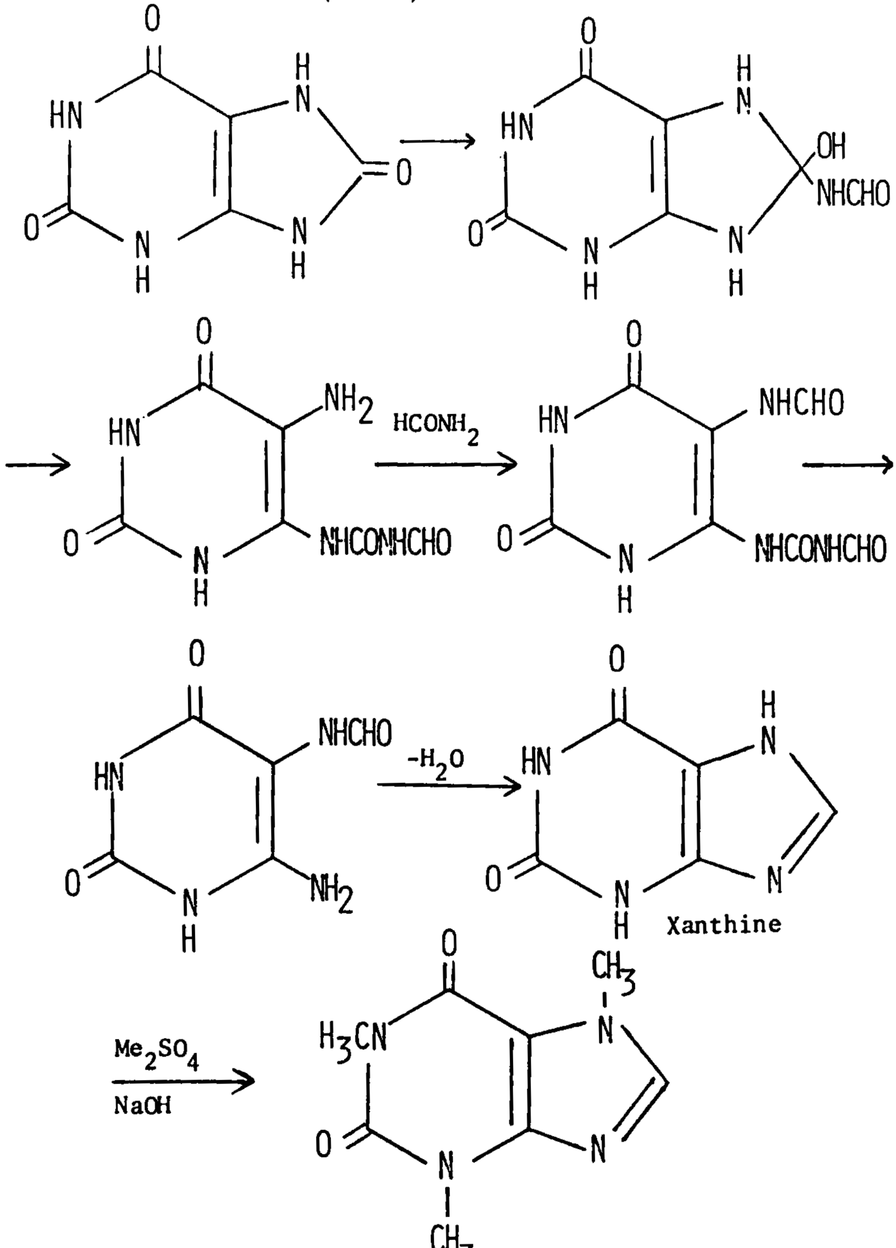
4.1 Route 1

Uric acid with methyliodide gave 1,3,7-trimethyluric acid, which was converted into 8-chloroderivative and its subsequent dehalogenation afforded caffeine (38-40).



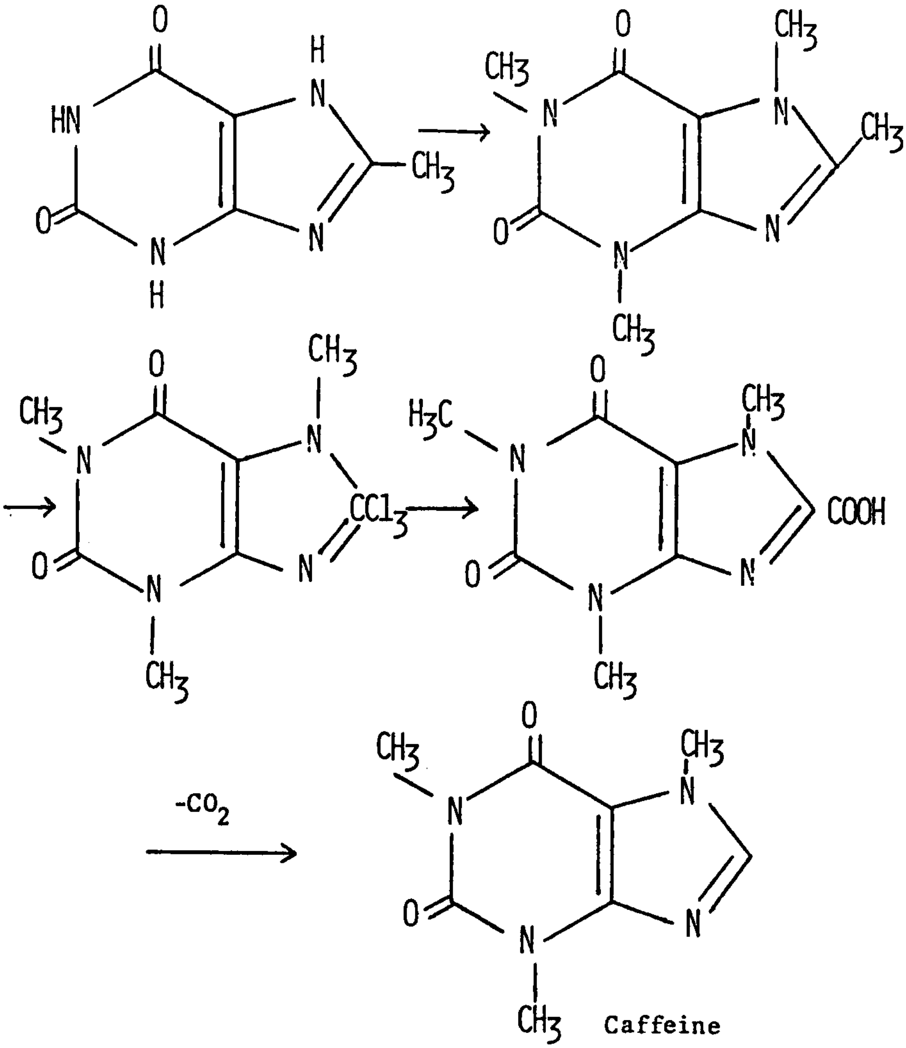
4.2 Route 2

Caffeine has also been synthesised from uric acid via another route (41-42).



4.3 Route 3

A different route for the preparation of caffeine from uric acid has been reported (43). Uric acid is first converted into 8-methylxanthine by the action of acetic anhydride (44-46), a process which involves the intermediate formation of a diacetyl derivative. The secondary amino groups are then methylated in an alkaline solution to give 1,3,7,8-tetramethylxanthine. Finally, the methyl group originally introduced in the 8-position is eliminated (47).



Caffeine

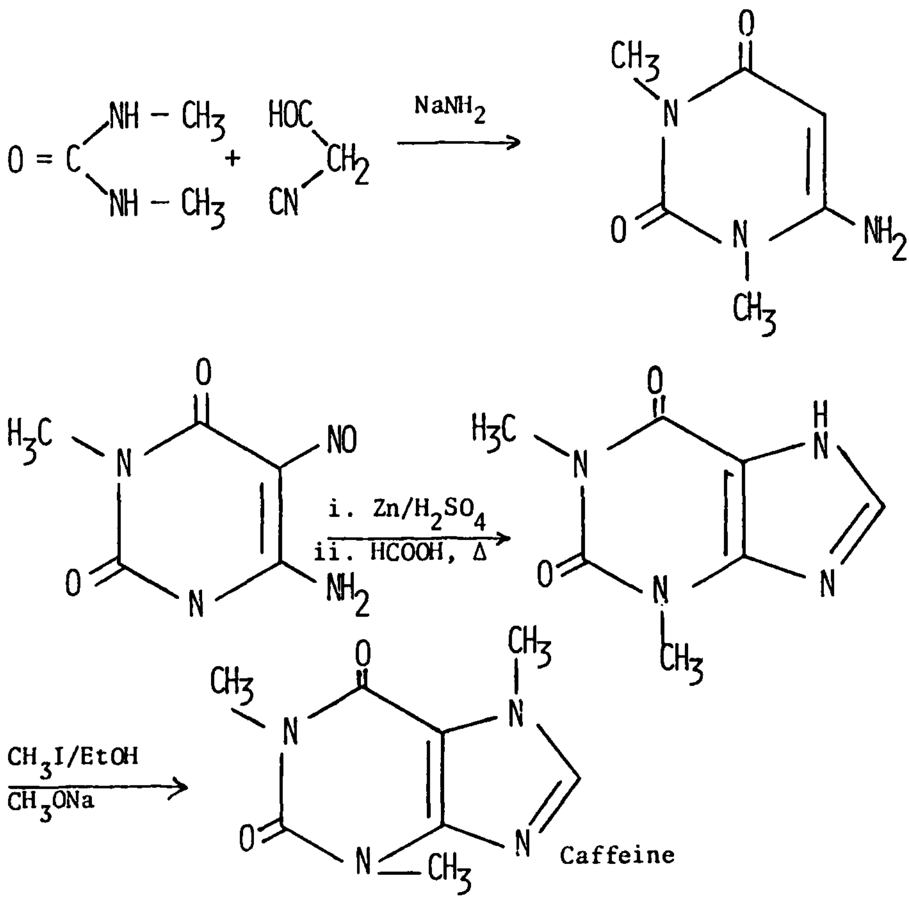
4.4 Route 4

Alkylation of xanthine derivatives has also been carried out for the synthesis of caffeine. Methylation of theobromine gives caffeine (48-50), while 1-methylxanthine has also been converted to caffeine via theophylline (48,49,51,52). 1,7-Dimethylanalogue (paraxanthine) on alkylation affords caffeine (53), while xanthine on treatment with an excess of methylating agent gives rise to a caffeine derivative (41,49,54,55).

4.5 Route 5

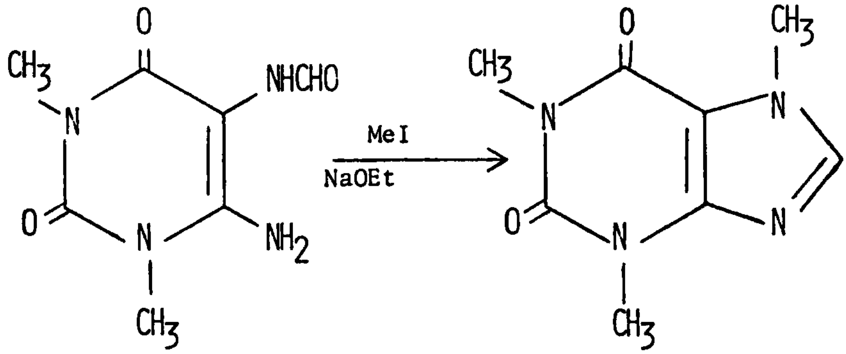
Caffeine is usually commercially synthesized by Traub's methods; (A and B, given below:) (56)

(A)



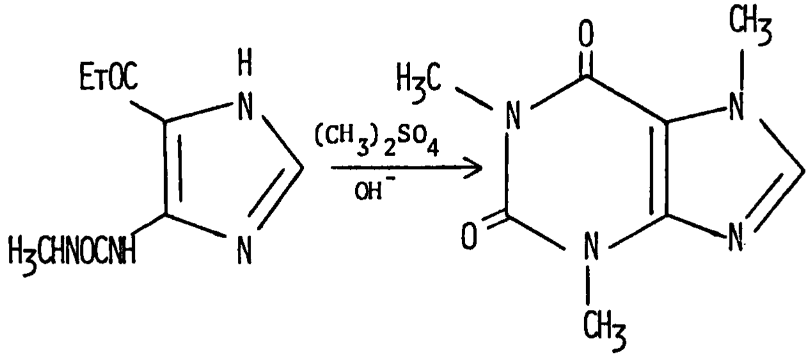
Caffeine

(B) In this method 4-amino-5-formamido-1,3-dimethyluracil is cyclised and methylated in one step in sodium ethoxide containing methyliodide to afford initially theophylline and subsequently caffeine (57).



4.6 Route 6

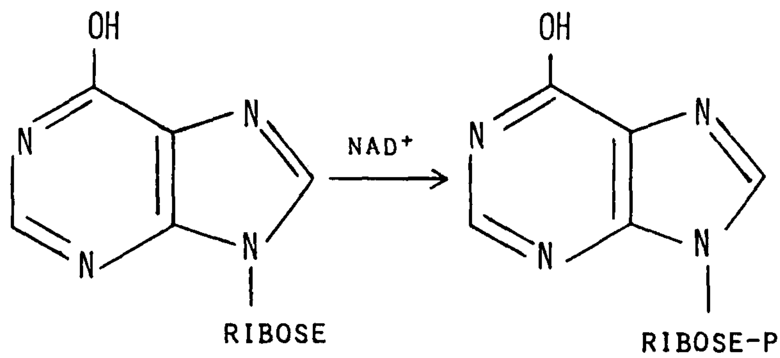
It involves ring closure of 5-ethoxycarbonyl-1-methyl-4-(N-methyl) ureidoimidazole, and subsequent methylation in alkaline medium gives caffeine (58).



Caffeine

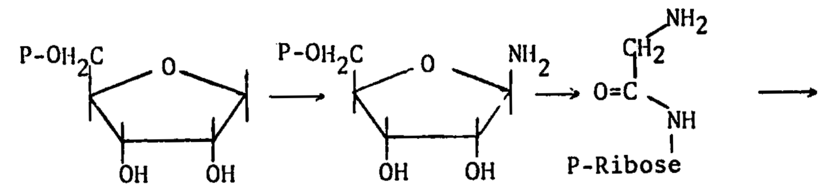
5. Biosynthesis

It has been reported (59-62) that biosynthesis of purine ring system probably proceeds in nearly the same manner in all organisms as shown in Scheme I. Initially, this pathway was proposed for micro-organisms and animals but recent evidence suggests that it may also hold good for higher plants. Inosine monophosphate is the first compound in this biosynthetic pathway to contain a complete purine ring system (59) and it has a central position in the purine metabolism. It can be converted to xanthosine monophosphate in an NAD^+ dependent reaction as shown below:

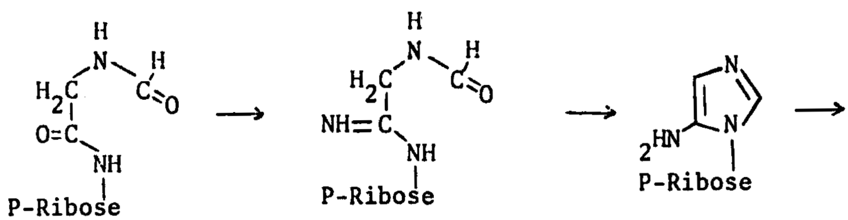


Inosine monophosphate Xanthosine monophosphate

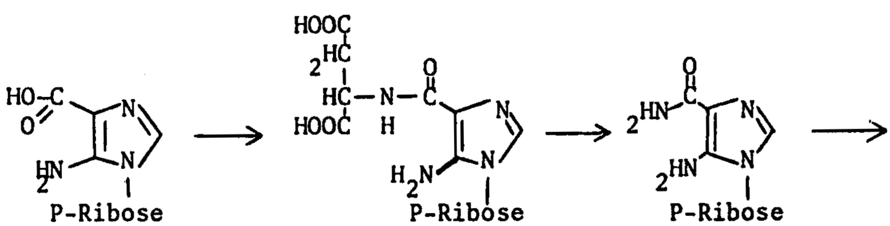
Xanthine, which originates from xanthosine monophosphate by elimination of phosphate and ribose, is the starting material for formation of caffeine and other purines. In the plant *Coffea arabica*, the xanthine is converted, to theobromine and to caffeine, either via N^3-methylxanthine



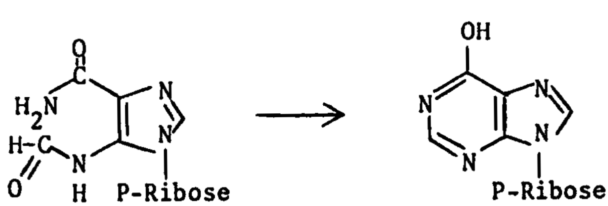
5-Phosphoribosyl -1- pyrophosphate | 5-Phosphoribosyl-1- amine | Glycinamide ribonucleotide



α-N-Formylglycinamide-ribonucleotide | N-Formylglycinamide ribonucleotide | 5-Aminoimidazole-ribonucleotide



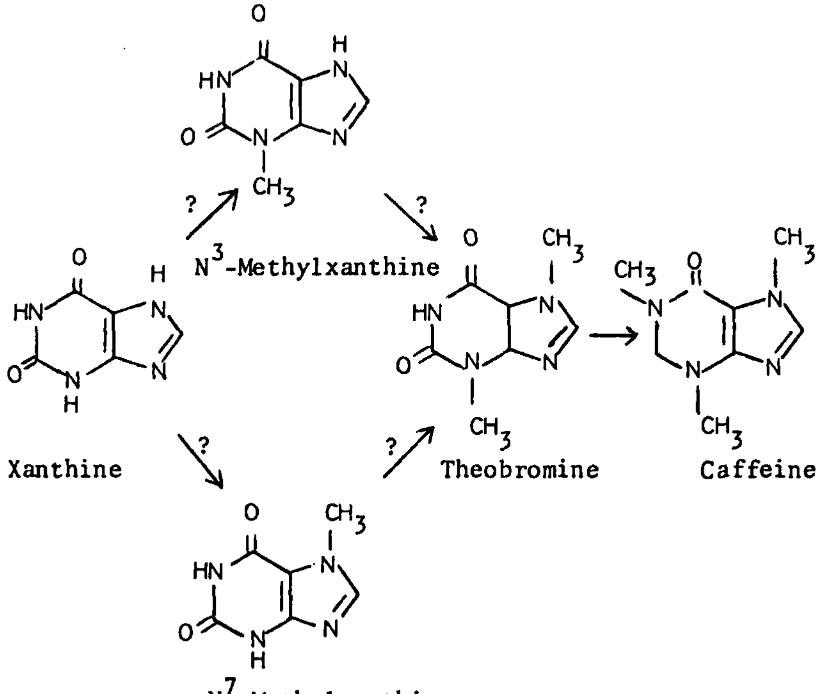
5-Aminoimidazole-4-carboxylic acid ribonucleotide | 5-Aminoimidazole-4-N-succinocarboxamide ribonucleotide | 5-Aminoimidazole-4-carboxamide ribonucleotide



5-Formamidoimidazole-4-carboxamide-ribonucleotide | Inosine-5-monophosphate

Scheme I. Biosynthesis of inosine monophosphate.

or N^7-methylxanthine Scheme II. The methyl groups originate from methionine (63-65).



Scheme II. Formation of theobromine and caffine from xanthine.

6. Pharmacokinetics and Metabolism

The absorption of caffeine from the gastrointestinal tract is rapid but irregular (67,68). It is distributed in various tissues of the body in approximate proportion to their water content (63). The absorption of caffeine is pH-related, an increase in pH increases its absorption (67). Within the tissue, caffeine is rapdily broken down (69), involving metabolite reactions, such as N-demethylation and oxidation, and ring cleavage (67). The drug metabolising enzymes of the liver are stimulated following the ingestion of large amount of caffeine (67).

The degree of caffeine degradation and degradation products excreted in the urine of different species seems to vary

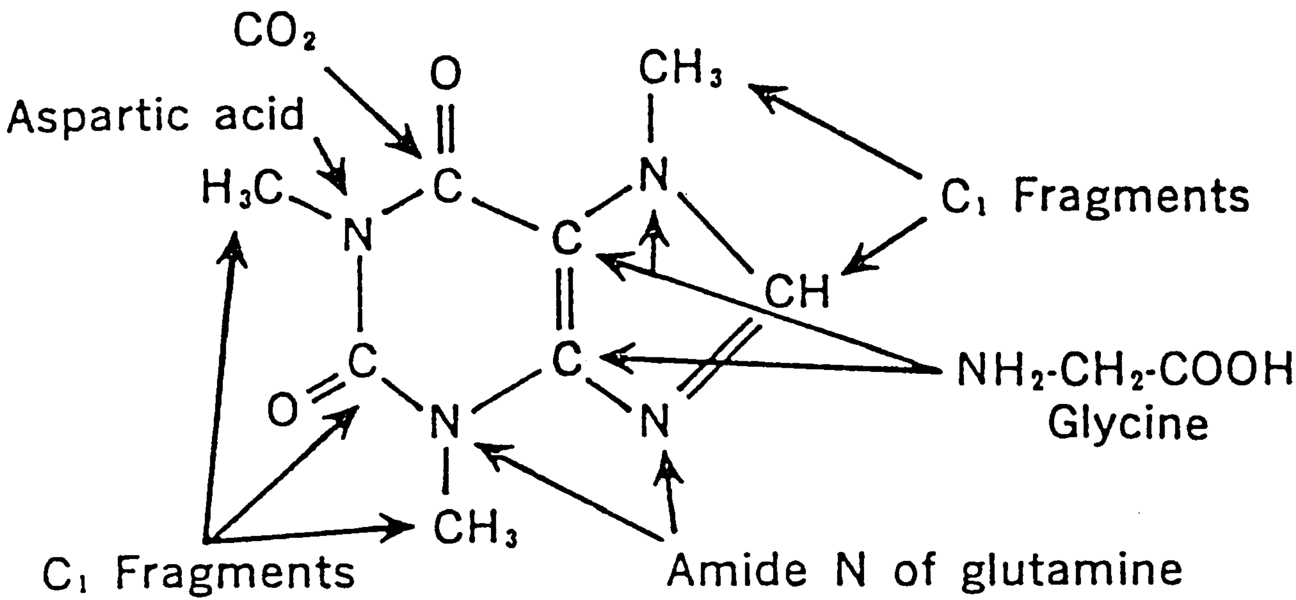


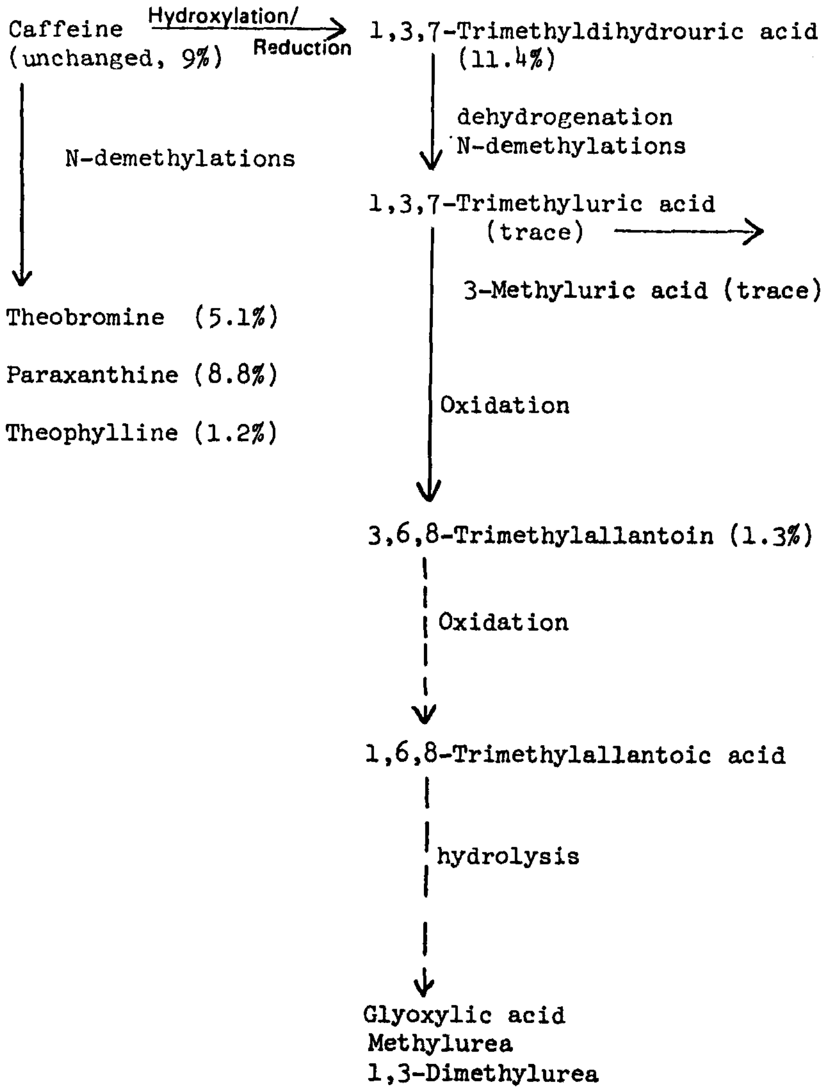
Fig. 12: Origin of various atoms in the biosynthesis of caffeine.

considerably (70). Blood level concentration in four animal species, following oral administration of 25 mg/kg [1-C^{14}] caffeine, have been reported by Burger (70), (Table 10). The plasma half-life for humans is between 4 to 10 hours (67).

Table 10. Plasma Concentration of [1-C^{14}] Caffeine in Different Animal Species.

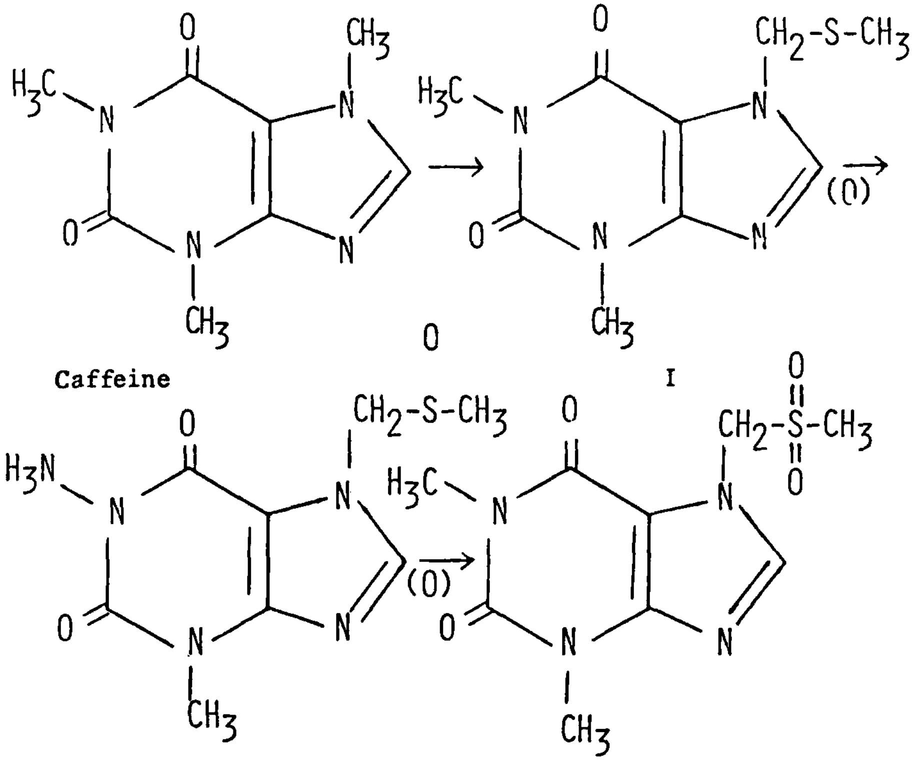
	Rat	Hamster	Rabbit	Rhesus monkey
Half-life (h)				
Radioactivity	5.4	3.5	11.0	19.0
Caffeine	2.8	3.1	3.7	2.8
Absorption half-time (h)				
Radioactivity	0.1	0.5	0.7	0.9
Caffeine	0.1	0.1	0.7	-
Peak plasma concentration of caffeine (μg/ml).	18	18	22	13

Caffeine metabolism in the rat liver slices and postnatal developing rats has been studied (71). Rao et al,(1973) have proposed a biotransformation route for caffeine in rats (72) (Scheme III).



Scheme III. Biotransformation of caffeine in rat.

An interesting sulphur containing metabolite of caffeine was isolated from the urine of mouse, rat, rabbit and horse (73), and is shown in Scheme IV.



Caffeine I

II III

Scheme IV. The sulphur metabolite of caffeine.

In case of humans, usually 45% of a dose is excreted in urine in 48 hours as 1-methylxanthine (67) and 1-methyluric acid (67,74). Other breakdown products excreted in the urine include, theophylline, 1,7-dimethylxanthine, 7-methylxanthine and 1,3-dimethyluric acid, along with some unchanged caffeine (67).

Further studies on human metabolism of (1-methyl-^{14}C) and (2-^{14}C)caffeine have been reported (75,76). Radiolabelled caffeine was administered (75) orally at 5 mg/kg to adult, male volunteers. Blood, saliva, expired CO_2, urine, and feces were analysed for total radiolabelled equivalents of

caffeine and its metabolites. High-performance liquid chromatography (HPLC) was the main technique used for the separation of caffeine and its metabolites with quantitation by liquid-scintillation counting. The half-life of caffeine in both serum and saliva was approximately 3 hrs., with the amount of caffeine in saliva samples almost 65 to 85% of that found in serum samples. The main metabolites found in serum and saliva were the dimethylxanthines, paraxanthine, theophylline and theobromine (77). It has been reported (76) that demethylation of the 3-methyl groups seems to be the most important pathway in man, and the plasma concentration of the dimethylxanthines depend on their urinary excretions as well as their own metabolism (76). Effect of caffeine on alcohol metabolism(78), basal metabolism (75) and its effect on exercise performance have also been studied (79).

Caffeine metabolism in sheep has also been studied (80-82).

7. Methods of Analysis

7.1 Elemental Analysis

The elemental composition of caffeine is: (83)

Element	% Theoretical
C	49.48 %
H	5.19 %
N	28.85 %
O	16.48 %

7.2 Identification Tests

1. Addition of tannic acid solution to caffeine solution gives a precipitate, which dissolves on further addition of the reagent (84).

2. Addition of iodine solution and hydrochloric acid to caffeine solution gives a brown precipitate, which neutralises on addition of sodium hydrochloride (84).

3. Reaction of caffeine with potassium chlorate in hydrochloric acid, and subsequent exposure to ammonia, gives purple colour, which disappears on addition of a solution of a fixed alkali (85).

4. Addition of gold chloride solution to caffeine solution affords small rods (86).

5. Addition of mercuric chloride solution to caffeine solution gives long needles (86).

6. Comparison of the infrared spectrum of the caffeine sample with that of a reference standard is also employed (85).

7.3 Titrimetric

Several methods have been reported for the analyses of caffeine by acid-base titration procedures, either by using different indicators (87-100) or by potentiometric methods (101-108), for the end-point detection.

An iodometric titration method (88) was developed for the determination of caffeine. It is mixed with H_2SO_4 (1:1) and IBr solution, and the mixture is diluted to 100 ml. After filtration, to a portion of the filtrate is added KI solution, and the equivalent amount of iodine liberated is titrated with $Na_2S_2O_4$, using starch solution as indicator. A blank is also carried out at the same time.

Collado et al., have reported a method (87) for the determination of caffeine, phenazone, phenacetin and phenobarbitone, in analgesic tablets. For the estimation of caffeine, phenazone is precipitated with picric acid and filtered off, and caffeine is determined iodimetrically in acid solution.

Caffeine can be determined iodimetrically from ice-cream and cocoa admixtures (109,110). 40 g of the sample is heated with 10 ml of water, and 1 ml of 15% aqueous NaOH, on a boiling water bath for 30 minutes. The mixture is cooled and 3 ml of 30% $Pb(NO_3)_2$ is added. It is diluted to 100 ml with water and is filtered. 10 Ml of this sample is used to determine caffeine titrimetrically.

The U.S. Pharmacopoeial method (108) consists in dissolving about 400 mg of finely powdered caffeine in 40 ml of acetic anhydride. After mixing with 80 ml of benzene it is titrated with 0.1N perchloric acid, determining the end-point potentiometrically.

Caffeine has been assayed for another non-aqueous titration procedure (93). It is dissolved in warm benzene and after cooling, couple of drops of a suitable indicator, such as Sudan IV or Nile blue A, are added before titration with 0.1N $HClO_4$ in glacial acetic acid.

Caffeine in cacao shells has also been determined (100) by non-aqueous titrimetric method. About 2 g of the ground and de-fatted sample is mixed with magnesium oxide and water. The mixture is heated on a water bath for 30 minutes and then it is extracted into chloroform in a soxhlet apparatus. The chloroform extract is dried at 105°C for 25 minutes and the residue is first titrated for total theobromine and caffeine, and then for theobromine only.

7.4 Spectrophotometric

7.4.1 Colorimetric

A sample containing 100 mg is dissolved in 100 ml of water and is filtered (111). An aliquot of 5 ml is diluted to 25 ml and 1 ml of dilute hydrochloric acid, and 1 ml of 10% molybdophosphoric acid are added to it. The mixture after heating on a water bath is chilled in ice and centrifuged. The precipitate is washed and dissolved in 25 ml of acetone and the extinction is measured at 440 mμ. The results with compounded tablets show recoveries of caffeine, 100.2% of theoretical amount.

Corte's method (112,113) consists in liberating caffeine with concentrated H_2SO_4, followed by extraction with $CHCl_3$. It is then estimated by a modified colorimetric method involving formation of its periodide (114).

In another method (115) caffeine (110 to 400 μg) is dissolved in a freshly prepared solution of 0.5 ml of acetylacetone and 5 ml of 2N NaOH. This mixture is heated at 80°C, and after cooling, a solution of p-dimethylaminobenzaldehyde and 20 ml of conc. HCl are added. The mixture is further heated at 80°C, and after cooling, 10 ml of water is added and extinction of the resulting blue solution is measured at 615 mμ.

Efforts to optimise conditions (116) have resulted in an improved colorimetric method (117) for the estimation of caffeine.

A small amount of caffeine is dissolved in a mixture of 3% aqueous acetic acid solution, and 10% aqueous pyridine solution. The mixture is then made up to 18 ml with water and 2 ml of NaOCl solution is added. Add 2 ml of 0.1N $Na_2S_2O_3$, followed by 3 ml of 1N NaOH, solution and dilute to 50 ml with water. The extinction, in 4 cm cell is measured at 460 mμ.

Iodine has also been employed as a colorimetric reagent for the determination of caffeine (118). 3N iodine (1 ml) solution is mixed with 1% solution of caffeine (5 ml) followed by 50% H_2SO_4 (0.5 ml) and is set aside for 10 minutes. The precipitate is filtered and washed before dissolving it in acetone. Its extinction is measured at 525 mμ.

A colorimetric method for the determination of caffeine in pharmaceutical preparations has been developed (119-121). The test solution is treated with an equal volume of 10N NaOH at 120°C to convert caffeine into caffeidine. It is then coupled with diazotised sulphanilic acid and the extinction is measured at 451 mμ. Other drugs present in usual caffeine pharmaceutical preparations do not interfere.

Caffeine present in beverages can also be estimated colorimetrically (121-122). It is extracted from the beverage by the known (123) method and is treated with 2% solution of malonic acid in acetic anhydride. The mixture is heated at 90°C, and after cooling it is made up to 25 ml with methanol. The extinction of the resulting greenish-yellow solution is measured at 430 nm.

7.4.2 Ultraviolet

Spectrophotometric methods for the estimation of caffeine in pharmaceutical preparations and its mixtures with other drugs and compounds have been developed (124-129). These invariably involve

preliminary separation of caffeine, before its spectrophotometric estimation.

An indirect spectrophotometric method for the determination of caffeine in quaternary mixtures such as NaOBz, p-EtoC_6H_4NHAc, aminopyrine and caffeine has been reported (129). The percentage of these components can be estimated by a compact calculation scheme after determination of extinction coefficients at the given wavelengths.

Several ultraviolet spectrophotometric methods have been developed for the estimation of caffeine in coffee and decoffeinated coffee (130-136), tea (137-141) and beverages (142-146).

Caffeine can also be determined in biological fluids spectrophotometrically (147). 0.1N-NaOH is used to extract caffeine, and a mixture of solid NaCl and Na_2SO_4 is added to enhance the extraction of caffeine into ether-chloroform. The caffeine is then transferred to an aqueous acidic solution, the solution in the reference cell is made strongly acid, and the contents of the sample cell are adjusted to pH 1.3.

7.4.3 Infrared

Infrared spectrophotometric method has been used (148,149) for the analysis of caffeine. A procedure for the determination of caffeine in pharmaceutical preparation containing aminopyrine and phenacetin has been reported (148), and the region 650 to 400 cm^{-1} of the spectrum, in KBr disc, was used for rapid and simultaneous determination of the three components.

7.4.4 Nuclear Magnetic Resonance

A simultaneous quantitative NMR method for the analysis of aspirin, phenacetin and caffeine in pharmaceutical preparations has been reported (150). The method involves comparing the integrals of the CH_2-signal (at 6 ppm) of piperonaldehyde, used as an internal standard, with those of aspirin methyl singlet (2.3 ppm),

phenacetin, ethyl triplet (at 1.3 ppm) and caffeine methyl singlet (at 3.4 ppm). The average percent recoveries and standard deviations were 95.61 ± 0.37, 96.34 ± 0.47 and 101.26 ± 1.46 for aspirin, phenacetin and caffeine respectively.

NMR-shift technique in forensic chemistry for the analyses of mixed samples of caffeine has been reported (151). NMR studies of metal porphyrin caffeine complexes (151) and association of caffeine with sodium benzoate have also been reported (152,153).

7.4.5 Mass Spectrometric

A new and simple analytical method using direct-inlet chemical ionisation mass spectrometry, has been reported (154) for simultaneous determination of caffeine and other components of an anti-cold drug. These components were detected as quasimolecular ions, and were quantitated by using an accumulation program.

In another method (155) B^2E = constant, linked scan has been used as a tool for quantifications with reversed-geometry mass spectrometers. D-labelled analogs may be used as internal standards, thus providing very simple clean-up procedures. The method is applicable to caffeine present in beverages.

Caffeine was analysed together with other drugs like acetyl salicylic acid and phenacetin by mass spectrometry (156).

7.4.6 Photo-nephlometric

Caffeine can be estimated in tea by using photo-nephlometer (157). A calibration curve is obtained by mixing 2 ml of 0.01M sodium tungstophosphate, 4 ml of 2.5M HNO_3 and 0.5 to 2.5 ml of 0.001M caffeine solution. It is then diluted to 50 ml and examined in a photo-nephlometer. Other compounds present in tea do not cause any interference. With some modifications, this

procedure can be used for the determination of caffeine in presence of various drugs.

7.5 Coulometric

Kalinowska has used coulometric technique for the estimation of caffeine (158-162). About 0.05 g of caffeine is dissolved in hot water, and then mixed with 6 ml of 30% KOH. The mixture is further heated in a boiling water bath, after cooling and dilution with water, it is neutralised with hydrochloric acid. About 1 ml of this solution is used for the determination of caffeine with coulometrically generated chlorine, with a current of 5 mA. The end-point is determined by the dead-stop end method. The coefficient of variation was about 0.5%.

7.6 Polarographic

Caffeine, although not reducible at dropping-mercury electrode can be determined polarographically after its oxidation with bromine (163). The wave heights of the resulting substituted parabanic acids are then evaluated. This method is quite sensitive and the results obtained are within ± 3%. Barbiturates, acetylsalicylic acid, ephedrine, codeine, papaverine and digitoxin do not interfere; however, phenazone, phenacetin and amidopyrine should be removed before the reaction.

In another method (164), the caffeine is analysed by anodic differential phase voltametry at a glassy C electrode, the detection limit is 0.5 ppm at pH 1.2. Oxidation products of caffeine are identified by cathodic differential pulse polarography.

7.7 Ring-Oven (Micro)

A relatively specific colour reaction between chloroform extract of caffeine, alkaline acetylacetone solution and acid p-dimethyl aminobenzaldehyde solution, has been used for the micro determination of air-borne particles of caffeine. It is possible to determine 0.5 μg of caffeine by this method with a mean error of ± 3% (165).

Table 11. Parameters Used for Paper Chromatography of Caffeine

No.	Support	Developing Solvent	Detection	Ref.
1.	S & S. 2043 Paper impregnated with ethanolic salicylic acid (0.3 g in 30 ml) and dried.	Consisted of salicylic acid (0.3 g) dissolved in butanol (30 ml); the solution being treated dropwise with H_2O, to the first turbidity.	Chromatogram immersed in 1% $AgNO_3$ dried and immersed in 0.5% aqueous $K_2Cr_2O_7$.	169
2.	Whatman DE20 Anion-exchange paper.	Develop with 0.2N-aq. ammonia for 105 minutes.	UV light	170
3.	Whatman No. 4 paper	Mobile phase was a xylene-tetralin-n-amyl alcohol mixture and the stationary phase was water acidified to pH 3.2.	--	171

7.8 Radioactive Isotopes

Caffeine can be estimated (166) by means of isotope-dilution analysis with [1-^{14}C] caffeine. It is prepared by methylation of theobromine with [C^{14}]methyl-iodide. About 10 mg of the purified sample is subjected to combustion in Baker's apparatus, using the Van Slyke-Folch reagent. The liberated CO_2 is absorbed in 0.25N-NaOH and 1.88% $BaCl_2$ solution is added. The precipitated $BaCO_3$ is filtered off and its specific radioactivity is measured with a Geiger-Muller tube. 30-80 Mg of caffeine, even in presence of acetylsalicylic acid, theobromine, aminophenazone or phenacetin give satisfactory results.

Noakes has reported (167) another radiocarbon measurement method for the estimation of natural-product purity in case of caffeine.

7.9 Radiochemical

Caffeine was also analysed by using radioactive tracer techniques (168). It is precipitated from the solution of the sample in hydrochloric acid with P^{32}-labelled phosphomolybdic acid, and after filtration, the tracer I-phosphomolybdate is dissolved in acetone and collected in a sample glass holder. The activity of each precipitate is determined and the amount calculated from a standard curve. The method is simple and gives reproducible results with small amount of samples.

7.10 Chromatographic Methods

7.10.1 Paper Chromatography

Some paper chromatographic systems used for the determination of caffeine have been summarised in Table 11.

7.10.2 Column Chromatography

Various column chromatographic systems used (175-182) for the determination of caffeine are presented in Table 12.

In another method (182) column chromatographic technique has been employed initially for the

Table 12. Summary of Conditions Used for the Column Chromatography of Caffeine

	Support	Eluent	Detection	Sample	Ref.
I.	Celite 545 and 4N H_2SO_4 covered by a layer of a mixture of celite 545 and N aq. $NaHCO_3$.	-	-	-	172
II.	Basic aluminuim oxide	Mixture of $CHCl_3$ and diethyl ether.	-	Pharmaceuticals and coffee	173
III.	Alumina (previously activated at 800°C for 6 hrs.) in a 100 ml burette.	(i) $CHCl_3$ (ii) H_2O	(i) Extinction of the eluent in $CHCl_3$ measured at 257, 277 and 297 mμ. (ii) For determination in aqueous solution the extinction is measured at 250, 273, and 296 mμ.	Coffee and its mixtures	174
IV.	(i) Celite and (ii) Column of celite containing 4N-H_2SO_4.	(i) Etyyl ether (ii) $CHCl_3$	276 mμ	Beverage and tablets.	175

Continued Table 12.

	Support	Eluent	Detection	Sample	Ref.
V.	MgO-Celite 545 (1:1, w/w)	H_2O	272 mμ	Caffeine-containing and caffeine-free coffee	176
VI.	Celite 545	$CHCl_3$	276.5 mμ	Coffee and decaffeinated coffee	177
VII.	Dowex 50W-X2	40% aqueous methanol	273 mμ	Pharmaceuticals	178
VIII.	Multiple column containing segments, (a) Celite 545 (3 g) and 1% tartaric acid soln. (b) Celite 545 (3 g) and 12% H_2SO_4 soln. (3 ml), (c) Celite 545 (2 g) and 8.4% $NaHCO_3$ soln. (2 ml) and (d) Celite 545 (3 g) and 22.1% K_3PO_4 soln. (3 ml).	Elute phenacetin with ether-$CHCl_3$ (8:1) 50 ml. Dismantle the column elute aspirin from segment (c) and determine caffeine.	--	Pharmaceutical combination of aspirin, phenacetin, and caffeine	179

Continued Table 12.

	Support	Eluent	Detection	Sample	Ref.
IX.	Polyamide (0.5 g)	After passing perculate, the caffeine is washed with H_2O, 2 X 3 X 10 ml.	272 nm	Tea	180
X.	Silica gel (particle size 0.04 to 0.063 mm) in column (30 cm X 1 cm)	H_2O-methanol (19:1)	254 nm	Soft drinks and food	181

isolation of caffeine from a sample of decaffeinated coffee, followed by its titration with 0.01M $HClO_4$ using methyl violet as indicator.

7.10.3 Thin-Layer Chromatography

A summary of some of the TLC systems investigated for the analysis of caffeine are given in Table 13. Combined TLC - colorimetric/spectrophotometric methods for the determination of caffeine in pharmaceutical preparations and beverages, have been reported (183,184). The method is based on the spectrophotometric estimation of the quantitatively eluted caffeine from TLC plates after separation from accompanying substances. TLC and high-performance TLC (1985) followed by densitometry has also been employed for the determination of caffeine in drugs (186,187), beverages (185,188,189) and cola seeds (189). The solution from the aspirin-phenacetin-caffeine tablets is chromatographed on Whatman chemically bonded KC_{18} reversed-phase plates containing fluorescent phosphor and using 1:1 MeOH-0.5M NaCl system (186). Caffeine was then determined by using a densitometer.

7.10.4 Gas Liquid Chromatography

Gas liquid chromatographic methods have been employed for the estimation of caffeine, and variable parameters used for some of these are summarised in Table 14.

7.10.5 High Performance Liquid Chromatography

High pressure liquid chromatography HPLC method has wide application for the estimation of caffeine in host of different samples. A summary of variable parameters in a few cases is given in Table 15.

Table 13. Summary of Conditions Used for the TLC of Caffeine

Support	Mobile phase	Detection	Sample	Ref.
Silufol	-	UV 254 nm	Drugs	190
Kieselgel $60F_{254}$ (layer thickness 0.2 mm) or	-	Spectrophotometrically	Caffeine	191
Cellulose F_{254} (layer thickness 0.1 mm)	-	-		
Kieselgel GF_{254}	$CHCl_3$-cyclohexane-acetic acid (8:2:1)	UV 254 nm	Coffee	192
Silica gel G	Spray with 2% $HgCl_2$ solution containing 10 mg of methyl red per 100 ml. or Sprayed with 2% $HgCl_2$ solution dried and sprayed with KI solution.	-		193
Kieselgel 60	Chloroform-acetone (9:1)	Chromatograms evaluated by remission densitometry at 273 nm with a double beam	Blood	194

Continued Table 13

Support	Mobile phase	Detection	Sample	Ref.
Kieselgel F_{254}	Cyclohexane-acetone (4:5)	Determined by their quenching effect on the background fluorescence.	Drugs	195
Silica gel G	--	--	Drugs (powders)	196

Table 14. Summary of Conditions Employed for the GLC of Caffeine

Column	Support	Mesh	Length	Temp.	Flow (ml/min) Carrier gas	Sample	Ref.
3%, OV-17	Chromosorb W	-	-	210°C	Argon		197
Tenax GC or			0.3 m	250°C		Flavor analysis and beverages	198
5% OV17	Chromosorb WHP		1.0 m	200°C			
SE-30	Chromosorb G	80-100	2.0 m	180°C	N_2(30 ml/min) (flame ionisation detector).	Drug	199
Varaport 30	3% Dexsil 300	-	2.0 m	280°C or (310°C if sample contains papave-rine)	N_2(16 ml/min) (flame ionisation detector).	Drug	200
Chromo-sorb W	3.5% sili-cone SE-30		1.5 glass	190°C	N_2(55 ml/min) H-flame ionisation	Drug biological material	201

Continued Table 14.

Column	Support	Mesh	Length	Temp.	Flow (ml/min) Carrier gas	Sample	Ref.
Chromo-sorb W AW/DMCS	3% OV-17	100-120	(Stain-less-Steel) 2m X 0.125 in o.d.	200°C	N_2 gas (30 ml/min) flame ionisation detector	Plasma	202
Gas Chrom Q	3% OV-17	-	-	180°C	-	Drugs and beverages	203

Table 15. Summary of HPLC Conditions for the Determination of Caffeine

Column	Mobile phase	Flow (ml/min)	Retention time (min)	Detection	Sample	Ref.
Partisil 10 SCX	Aqueous solution of 15 mM potassium citrate, pH 3.0 and 10% (v/v) methanol	1.1 ml/min	-	U.V. (280 nm)	Tea and coffee	204
μ Bondapak C18, 10 μm particle size	MeOH+Water+HOAc (20+79+1)	-	-	U.V.	Cocoa and chocolate products	205
Bondapak C18	MeOH-0.1M, pH 7.0, citrate-phosphate buffer (20:80)	2 ml/min	10	U.V. (254 nm)	Black tea infusion	206
Corasil I or Corasil II	Heptane+ethanol (10:1)	300 p.s.i.		U.V. (270 nm)	Purine alkaloids	207
C18 (Radial Pak A) cartridge	Water+MeOH+HOAc (74:25:1)	3.0 ml/min		U.V. (280 nm)	Animal diets	208

Continued Table 15.

Column	Mobile phase	Flow (ml/min)	Retention time (min)	Detection	Sample	Ref.
Bondapak C18	0.025 M NaH_2PO_4 in MeOH+water (2:3) to pH 7 using NaOH	2 ml/min	0.48	U.V. (254 and 280 nm)	Drugs	209
Spherosil XOA 600 and XOA 800	Iso-octane+diiso-propyloxide+MeOH+triethylamine+water (34.93+49.51+14.58+0.20+0.78)	1 ml/min	Aprox. 4 min	-	Drugs	210
Silica gel (M&N Nucleo-sil 50-5.5 μM)	Methylene chloride: ethanol: water (936:47:17)	50 ml h^{-1}	-	U.V. (280 nm)	Caffeine	211
Phenyl/Cora-sil and Corasil II	Acetonitrile-water; 1,4 dioxan-water; methanol-water; 1-4-dioxan-aceto-nitrile-water and various mixtures of chloroform with methanol	-	-	-	Drugs	212

Continued Table 15.

Column	Mobile phase	Flow (ml/min)	Retention time (min)	Detection	Sample	Ref.
Zipax SCX	0.01M HNO_3	4 ml/min		U.V. (254 nm)	Coffee	213
--	5% Glacial acetic acid	-	-	U.V.	Beverages	214
Bond elut, C18 bonded silica	Chloroform	-	-	-	Beverages	215
µ Bondapak C18	Methanol-water (30:70)	1.0 ml/min	-	U.V. (273 nm)	Plasma	216
Bondapak C18	MeCN-Water (8:92)	-	-	-	Beverages	217
Spherisorb-ODS with gradiant elution	--	-	-	U.V. (272 nm)	Decaffeinated, instant coffee and other beverages	218
10-µM Partisil ODS-2	MeCN-H_2O (30:70)	-	-	U.V. (254 nm)	Appetite/suppressant formulations	219

Continued Table 15.

Column	Mobile phase	Flow (ml/min)	Retention time (min)	Detection	Sample	Ref.
YWG-CH200x 5 mm	50% soln. of MeOH in 0.002M K_2HPO_4 adjusted to pH 8.0 by H_3PO_4.	1.0 ml/min		U.V. (254 nm)	Drugs	220
Hypersil	5% Isopropanol dichloromethane	-	-	U.V. (280 nm)	-	221
Ion exchange resin, Dowex-AG 50W-X8(H^+)	25% aqueous ethanol	15 ml/hour	-	U.V. (254 nm)	-	222
Nucleosil-5 C18	$MeCN$-H_2O-$HOAc$ (13:87:1)	-	-	U.V. (254 nm)	-	223
Finepak gel-110 or Finepak SIL-C18	$MeOH$-NH_4OH (99:1) and $MeCN$-NH_4OH (99:1)	-	-	-	-	224

Continued Table 15.

Column	Mobile phase	Flow (ml/min)	Retention time (min)	Detection	Sample	Ref.
Bondapak C18/corasil and μ Bondapak C18	0.01M sod. acetate buffer, pH 5.0-MeOH-tetrahydrofuran (95:4:1)	3 ml/min	-	U.V. (254 nm)	Umbilical cord plasma	225
Radial-Pak C18 reversed phase column	Acetonitrile in 0.1 mol/L potassium phosphate buffer, pH 4.0 (9.5/90.5 by vol.).	-	-	-	Biological fluids (neonates)	226
Partisil 5ODS-3RAC or 5C8RAC (Radial-PAK μ Bondapak C18 cartridge, HS/5C18 or HS/3C18 or partisil 10-ODS-3	Containing H_2O, acetonitrile H_3PO_4 and NaOH with or without hexylamine	-	-	-	Various drugs samples including caffeine	227

Continued Table 15.

Column	Mobile phase	Flow (ml/min)	Retention time (min)	Detection	Sample	Ref.
Shodex D-814 (50 cm X 8 mm)	Aqueous 70% methanol at $40^{o}C$.	-	-	270 nm and by differential refractometry	Horse urine	228
Radial-PAK C18	0.1M-phosphate buffer (pH 4) - acetonitrile (181:19).	4 or 5 ml min^{-1}		254 nm	Caffeine and theophylline reonates	229
Micro packed fused-silica, used 10 and 5 cm columns were used.	-	-	-	U.V.	Caffeine, aspirin and phenacetin	230
μ Bondapak C18 (30 cm X 3.9 cm) with Bondapak C18/ Corasil guard column	10 m M-Na acetate buffer of pH 5 methanol-tetrahydrofuran (95:4:1).	3		254 nm	Metabolites in Umbilical cord plasma of bovine	231

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COCAINE HYDROCHLORIDE

FARID J. MUHTADI and ABDULLAH A. AL-BADR

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1. Description

1.1 Nomenclature

1.1.1 Chemical Names

- [1R - (exo,exo)]-3-(Benzoyloxy)-8-methyl-8-azabicyclo [3.2.1] octane-2-carboxylic acid methyl ester.
- (-) 3-(Benzoyloxy)-8-methyl-8-azabicyclo [3.2.1] octane-2-carboxylic acid methyl ester [1(R), 2(R), 3(S)].
- 3 β-Hydroxy-1 α H, 5 α H-tropane-2β-carboxylic acid methyl ester benzoate.
- 8-Azabicyclo [3.2.1] octane-2-carboxylic acid 3-(benzoyloxy)-8-methyl-methyl ester hydrochloride [1R-(exo-exo)].
- 2 β-Carbomethoxy-3β-benzoxytropane.
- Methyl-3 β-hydroxy-1 α H, 5 α-tropane-2 β-carboxylate, benzoate (ester) hydrochloride.
- (1R, 2R, 3S, 5S)-3-Benzoyloxy-2-methoxy carbonyl tropanium chloride.
- (1R, 2R, 3S, 5S)-2-Methoxycarbonyl-tropane-3-yl-benzoate.
- 2(R)-Carbomethoxy-3(S)-(-)-benzoxy-1(R)-tropane.

1.1.2 Generic Names

Cocaine; l-Cocaine; β-Cocaine; Benzoylmethylecgonine; Methylbenzoylecgonine; Ecgonine methyl ester benzoate.

Cocaine hydrochloride; Cocaine muriate; Neurocaine hydrochloride.

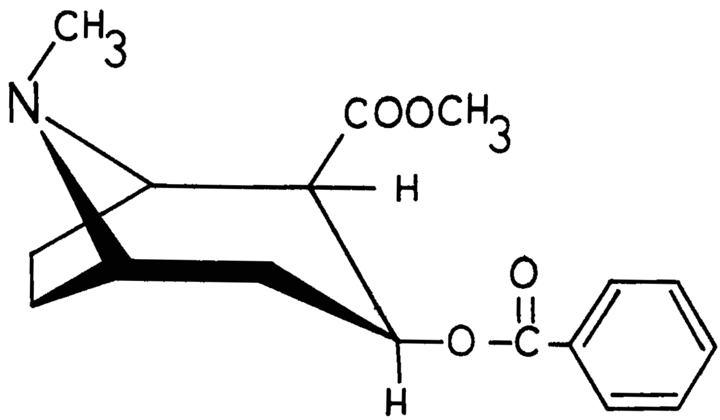
1.2 Formulae

1.2.1 Empirical

$C_{17}H_{21}NO_4$ for cocaine.

$C_{17}H_{22}NO_4Cl$ for cocaine hydrochloride.

1.2.2 Structural



The structure of cocaine was confirmed by the total synthesis of cocaine which was achieved by several authors (1-3).

1.2.3 CAS Registry Numbers

[50-36-2] for cocaine.
[53-21-4] for cocaine hydrochloride.

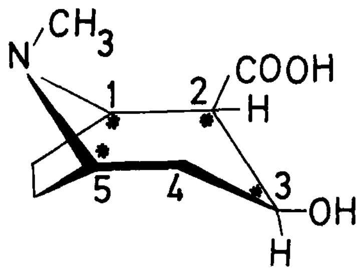
1.2.4 Wiswesser Line Notations

T 56 A ANTJ A - FVO1
GOVR - & GH LV (Cocaine hydrochloride) (4).

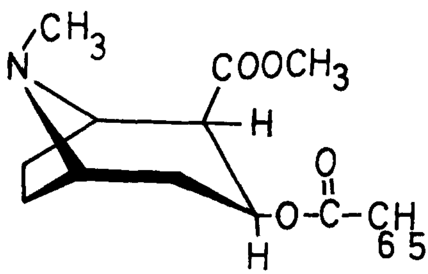
1.2.5 Stereochemistry

The ecgonine moiety of cocaine possesses four

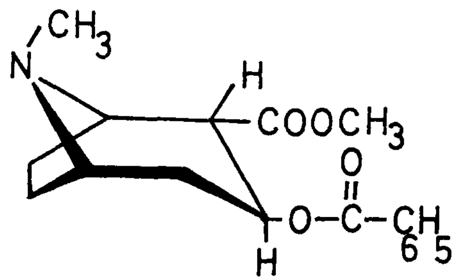
dissimilar chiral centres at C_1, C_2, C_3 and C_5 and so there are eight pairs of enantiomers possible.



Since, however, only the cis fusion of the nitrogen bridge is possible in practice, C_1 and C_5 therefore have only one configuration (the cis form), and so there are only four pairs of enantiomers actually possible, three pairs of which have been prepared synthetically (5).
The relative configurations of cocaine and Ψ-cocaine have been exclusively determined by chemical methods (6-11).

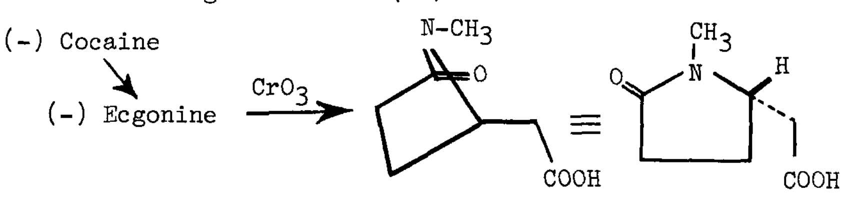


Cocaine



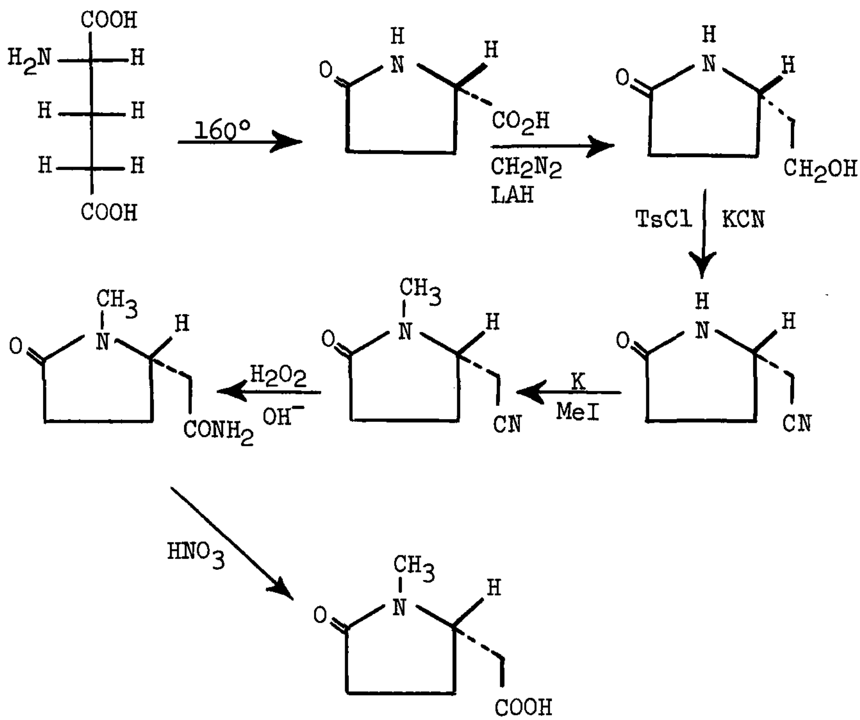
Ψ-Cocaine

The absolute configuration of (-)-cocaine was established by the following methods.
a) Correlation with L(+)-glutamic acid through ecgoninic acid (12).



(-)Ecgoninic acid

L(+) glutamic acid



(-) ecgoninic acid

b) X-ray crystallographic study of l-cocaine hydrochloride (13).
c) The stereoselective synthesis of dl-cocaine (3).

From the above data the absolute configuration of (-)-cocaine is presented in Fig. 0.

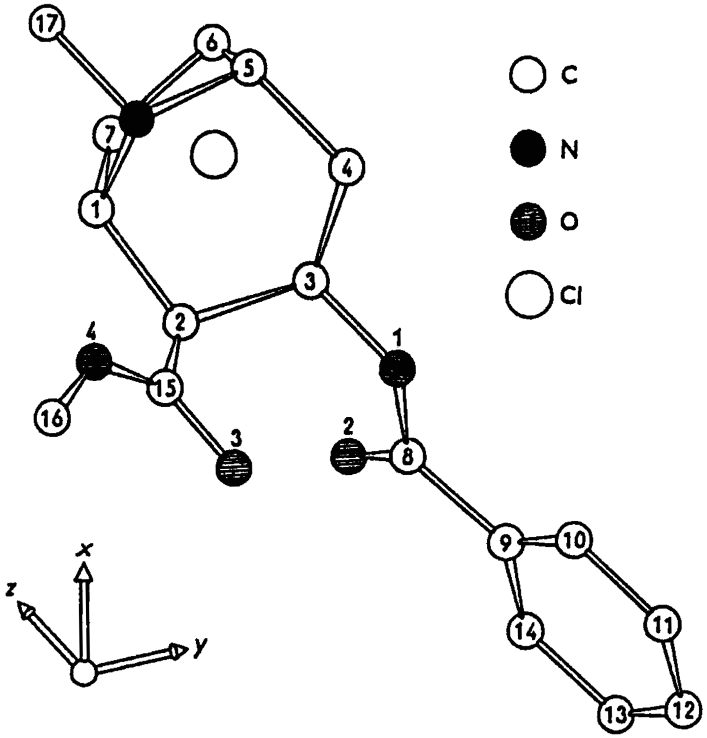


Fig.0 Perspective view of the molecule of cocaine (13).

1.3 Molecular Weight

303.35 (cocaine)
339.81 (cocaine hydrochloride)

1.4 Elemental Composition

C, 67.30%; H, 6.98%; N, 4.62%; O, 21.10%. (cocaine)

C, 60.08%; H, 6.53%; N, 4.12%; O, 18.83%; Cl, 10.43%. (cocaine hydrochloride)

1.5 Appearance, Color, Odor and Taste

Colorless crystals or a white crystalline powder, odorless, slightly volatile. It has a bitter taste, numbs tongue and lips. (Cocaine).

Colorless granule crystals, or a white crystalline powder, odorless and hygroscopic. It has a slightly bitter taste, numbs tongue and lips. (Cocaine hydrochloride).

1.6 Dissociation Constant

pKa at 15° = 5.59 (cocaine); pKa 8.4 (cocaine HCl)

1.7 Loss on Drying

When dried to constant weight at 80° loses not more than 0.5% of its weight (14).

2. Physical Properties

2.1 Melting Range

96 - 98°	(14)	cocaine
98°	(15)	cocaine
About 197°	(14)	cocaine hydrochloride
About 195°	(15)	cocaine hydrochloride

2.2 Solubility

One gram dissolves in 600 ml water, 270 ml water at 80°, 6.5 ml alcohol, 0.7 ml chloroform, 3.5 ml ether, 12 ml olive oil, also soluble in acetone, ethyl acetate and carbondisulfide (Cocaine).

One gram dissolves in 0.4 ml water, 3.2 alcohol, 2 ml hot alcohol, 12.5 ml chloroform, also soluble in glycerol and acetone. (Cocaine hydrochloride)

2.3 Optical Rotation

Cocaine

$[\alpha]D^{20}$ - 16° (C=4 in $CHCl_3$); $[\alpha]D^{18}$ -35°(50% alcohol).

$[\alpha]D$ - 79° to -81° (0.6 g in 2.5 ml of M hydrochloric and sufficient water to produce 25 ml) (14).

Cocaine hydrochloride

$[\alpha]D$ - 72° (C=2 aqueous solution pH 4.5)

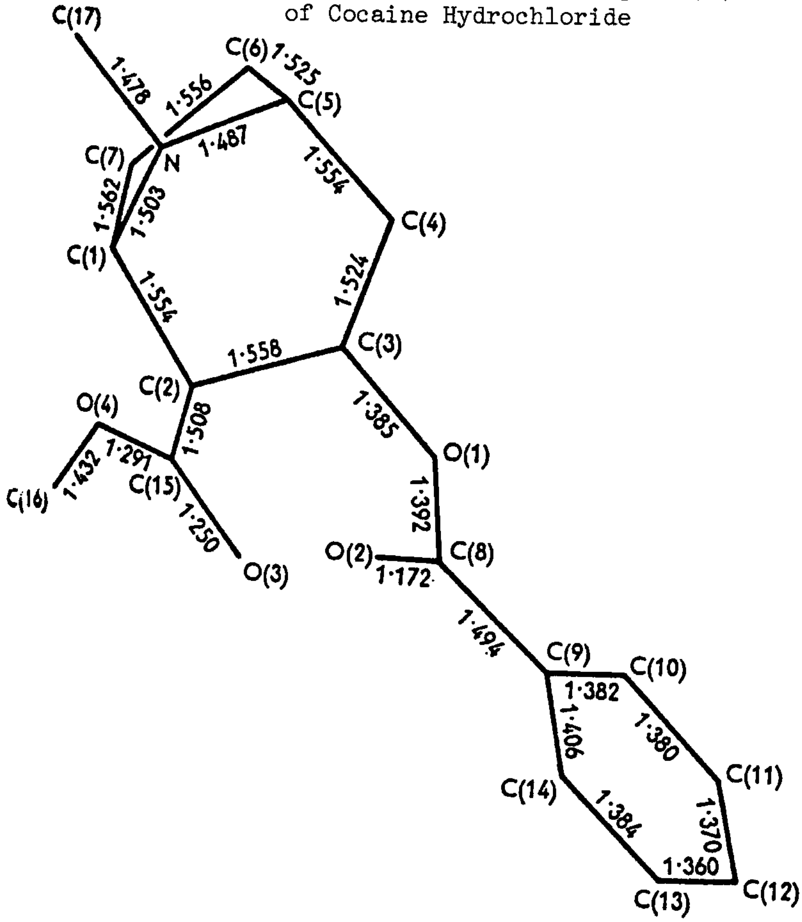
$[\alpha]D$ - 70° to -73° (2.5% w/v solution) (14)

2.4 X-ray Diffraction

The crystal and molecular structure of l-cocaine was determined by X-ray diffraction. Gabe and Barnes (13) have achieved this.

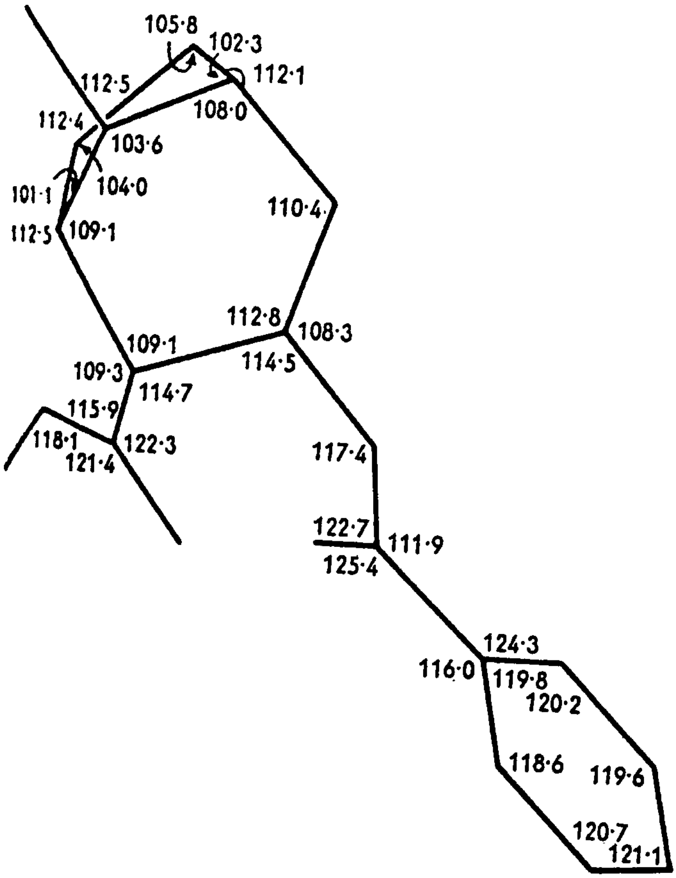
Three dimentional study of the hydrochloride and the hydrobromide salts revealed the crystals to be orthorombic with space group $P2_1 2_1 2_1$; for the hydrochloride, a = 7.633, b = 10.300, c = 21.459 A°; for the hydrobromide, a = 7.68, b = 10.68, c = 21.65 A°. The study also revealed that the stereochemical configuration of cocaine molecule agrees with that deduced from chemical evidence. The piperidine ring of the tropane nucleus has the chair form, with C_3 displaced less, and N displaced more than usual from the plane of the ring. The benzoxy side chain on C_3 is equatorial and the carbomethoxy side chain on C_2 is axial. Intramolecular bond lengths, bond angles and torsion angles of cocaine hydrochloride are tabulated in tables 1, 2 and 3 respectively.

Table 1. Intramolecular Bond Lengths (Å) of Cocaine Hydrochloride



$C_1 - N$	1.503	$C_1 - C_2$	1.554	$C_8 - C_9$	1.494
$C_5 - N$	1.487	$C_1 - C_7$	1.562	$C_9 - C_{10}$	1.382
$C_{17} - N$	1.478	$C_2 - C_3$	1.558	$C_9 - C_{14}$	1.406
$C_3 - O_1$	1.385	$C_2 - C_{15}$	1.508	$C_{10} - C_{11}$	1.380
$C_8 - O_1$	1.392	$C_3 - C_4$	1.524	$C_{11} - C_{12}$	1.370
$C_8 - O_2$	1.172	$C_4 - C_5$	1.554	$C_{12} - C_{13}$	1.360
$C_{15} - O_3$	1.250	$C_5 - C_6$	1.525	$C_{13} - C_{14}$	1.384
$C_{15} - O_4$	1.291	$C_6 - C_7$	1.556		
$C_{16} - O_4$	1.432				

Table 2. Bond Angles (°) of Cocaine Hydrochloride



$C_1 - N - C_5$	103.6	$C_4 - C_5 - C_6$	112.1
$C_1 - N - C_{17}$	112.4	$C_5 - C_6 - C_7$	105.8
$C_5 - N - C_{17}$	112.5	$C_1 - C_7 - C_6$	104
$C_3 - O_1 - C_8$	117.4	$O_1 - C_8 - O_2$	122.7
$C_{15} - O_4 - C_{16}$	118.1	$O_1 - C_8 - C_9$	111.9
$N - C_1 - C_2$	109.1	$O_2 - C_8 - C_9$	125.4
$N - C_1 - C_7$	101.1	$C_8 - C_9 - C_{10}$	124.3
$C_2 - C_1 - C_7$	112.5	$C_8 - C_9 - C_{14}$	116
$C_1 - C_2 - C_3$	109.1	$C_{10} - C_9 - C_{14}$	119.6

$C_1 - C_2 - C_{15}$	109.3	$C_9 - C_{10} - C_{11}$	120.2
$C_3 - C_2 - C_{15}$	114.7	$C_{10} - C_{11} - C_{12}$	119.6
$O_1 - C_3 - C_2$	114.5	$C_{11} - C_{12} - C_{13}$	121.1
$O_1 - C_3 - C_4$	108.3	$C_{12} - C_{13} - C_{14}$	120.7
$C_2 - C_3 - C_4$	112.8	$C_9 - C_{14} - C_{13}$	118.6
$C_3 - C_4 - C_5$	110.4	$O_3 - C_{15} - O_4$	121.4
$N - C_5 - C_4$	108	$O_3 - C_{15} - C_2$	122.3
$N - C_5 - C_6$	102.3	$O_4 - C_{15} - C_2$	115.9

Table 3. Torsion Angles (°) of Cocaine Hydrochloride (16).

$C_5 - N - C_1 - C_2$	72	$C_1 - C_2 - C_{15} - O_3$	-169
$C_5 - N - C_1 - C_7$	-47	$C_1 - C_2 - C_{15} - O_4$	17
$C_{17} - N - C_1 - C_2$	-166	$C_3 - C_2 - C_{15} - O_3$	-46
$C_{17} - N - C_1 - C_7$	75	$C_3 - C_2 - C_{15} - O_4$	140
$C_1 - N - C_5 - C_4$	-72	$O_1 - C_3 - C_4 - C_5$	-176
$C_1 - N - C_5 - C_6$	46	$C_2 - C_3 - C_4 - C_5$	-48
$C_{17} - N - C_5 - C_4$	166	$C_3 - C_4 - C_5 - N$	61
$C_{17} - N - C_5 - C_6$	-75	$C_3 - C_4 - C_5 - C_6$	-50
$C_8 - O_1 - C_3 - C_2$	77	$N - C_5 - C_6 - C_7$	-27
$C_8 - O_1 - C_3 - C_4$	-157	$C_4 - C_5 - C_6 - C_7$	89
$C_3 - O_1 - C_8 - O_2$	1	$C_5 - C_6 - C_7 - C_1$	-1
$C_3 - O_1 - C_8 - C_9$	179	$O_1 - C_8 - C_9 - C_{10}$	3
$C_{16} - O_4 - C_{15} - O_3$	8	$O_1 - C_8 - C_9 - C_{14}$	-178
$C_{16} - O_4 - C_{15} - C_2$	-178	$O_2 - C_8 - C_9 - C_{10}$	-179
$N - C_1 - C_2 - C_3$	-59	$O_2 - C_8 - C_9 - C_{14}$	0
$N - C_1 - C_2 - C_{15}$	67	$C_8 - C_9 - C_{10} - C_{11}$	-180
$C_7 - C_1 - C_2 - C_3$	52	$C_{14} - C_9 - C_{10} - C_{11}$	1
$C_7 - C_1 - C_2 - C_{15}$	178	$C_8 - C_9 - C_{14} - C_{13}$	180
$N - C_1 - C_7 - C_6$	28	$C_{10} - C_9 - C_{14} - C_{13}$	-1
$C_2 - C_1 - C_7 - C_6$	-88	$C_9 - C_{10} - C_{11} - C_{12}$	1
$C_1 - C_2 - C_3 - O_1$	172	$C_{10} - C_{11} - C_{12} - C_{13}$	-2
$C_1 - C_2 - C_3 - C_4$	47	$C_{15} - C_2 - C_3 - C_4$	-76
$C_{15} - C_2 - C_3 - O_1$	49	$C_{11} - C_{12} - C_{13} - C_{14}$	2

2.5 Spectral Properties

2.5.1 Ultraviolet Spectrum

The UV spectrum of cocaine hydrochloride in methanol (Fig. 1) was scanned from 190 to 400 nm using DMS 90 Varian Spectrometer. It exhibited the following UV data (Table 4).

Table 4. UV Characteristics of Cocaine Hydrochloride

λmax. nm	ε	A(1%, 1 cm)
202	4375	128.75
230	7136	210.0
272	433.3	12.75
282	390.8	11.50

Other reported UV spectral data for cocaine in ethanol λmax. 230, 274, 281 mu (17), for cocaine hydrochloride in water λmax. 233 and 274 mu (17).

2.5.2 Infrared Spectrum

The IR spectrum of cocaine hydrochloride as KBr-disc was recorded on a Perkin Elmer 580 B Infrared spectrophotometer to which an infrared data station is attached (Fig. 2). The structural assignments have been correlated with the following frequencies (Table 5).

Table 5. IR Characteristics of Cocaine Hydrochloride

Frequency cm^{-1}	Assignment
3020	CH (stretch)
2780-2500	$H_3C - N^+H$
1730	$\overset{O}{\overset{\parallel}{C}}-OCH_3$ (ester)
1715	$\overset{O}{\overset{\parallel}{C}}-O-$ (ester)
1600	C=C (aromatic)
1155,1028	C-O-C (ether)
730	monosubstituted aromatics

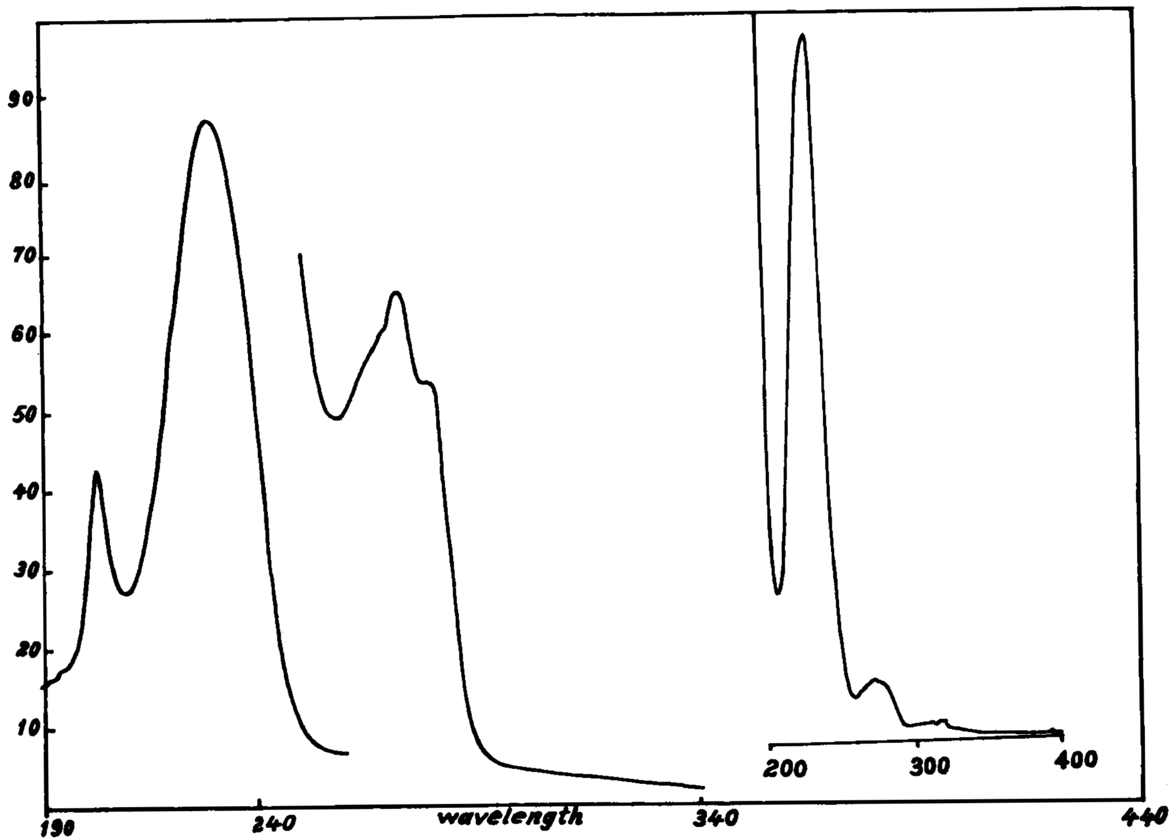


FIG. 1. THE UV SPECTRUM OF COCAINE HYDROCHLORIDE IN METHANOL

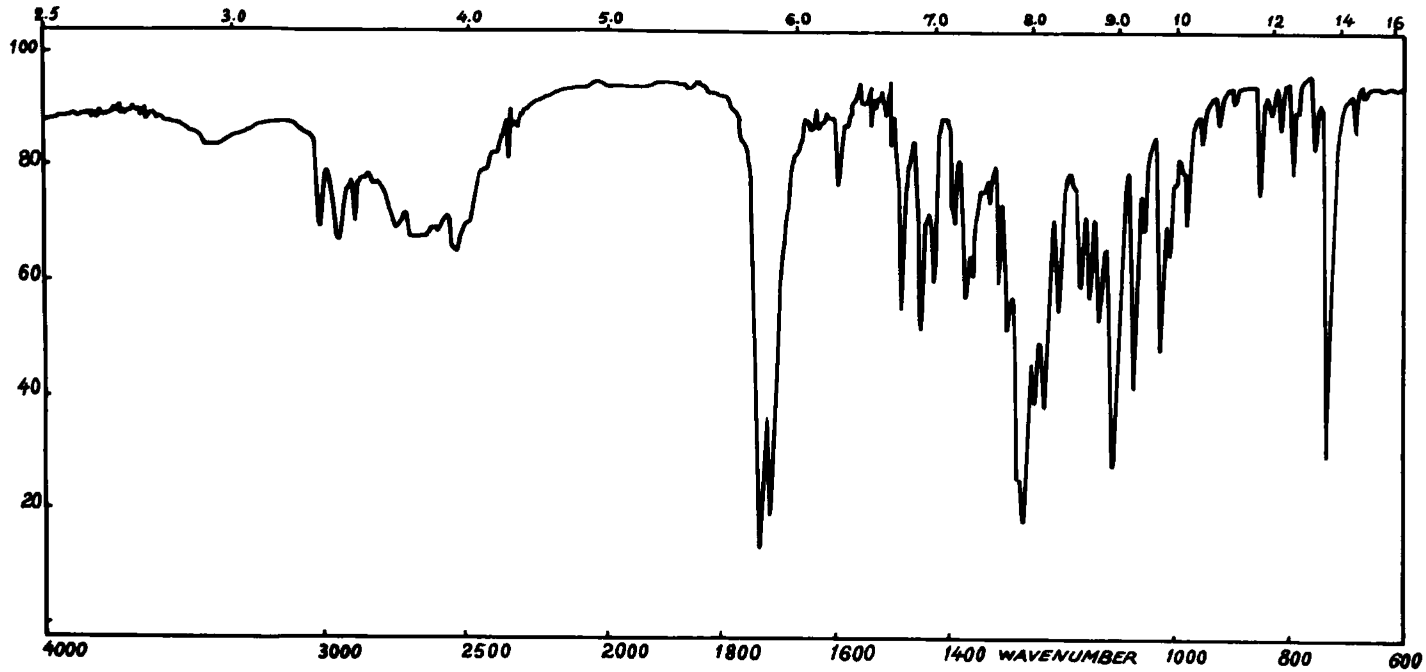


FIG. 2. THE IR SPECTRUM OF COCAINE HYDROCHLORIDE AS KBr DISC

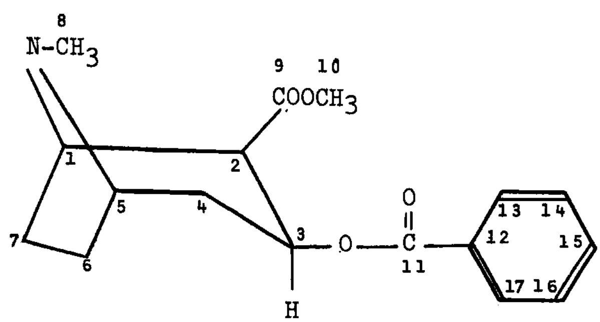
The IR exhibited the following other characteristic bands :-

1488, 1455, 1430, 1395, 1375, 1365, 1315, 1300, 1268, 1250, 1232, 1210, 1168, 1135, 1108, 1072, 1010, 980, 950, 855, 830, 815, 795, 755, 682 cm^{-1}.
Other IR data for both cocaine and cocaine hydrochloride have been also reported (4,17).

2.5.3 Nuclear Magnetic Resonance Spectra

2.5.3.1 Proton Spectra (PMR)

The PMR spectra of cocaine hydrochloride in D_2O and cocaine in $CDCl_3$ and in TFA (Trifluoroacetic acid) were recorded on a Varian T60A, 60 MHz NMR Spectrometer using TMS (Tetramethylsilane) as an internal reference. These are shown in Fig. 3 and Fig. 4 respectively. The following structural assignments have been made (Table 6).



PMR was used as a tool to confirm the configuration of both cocaine and pseudococaine (18) and provided evidence of the preferred confirmation (19).

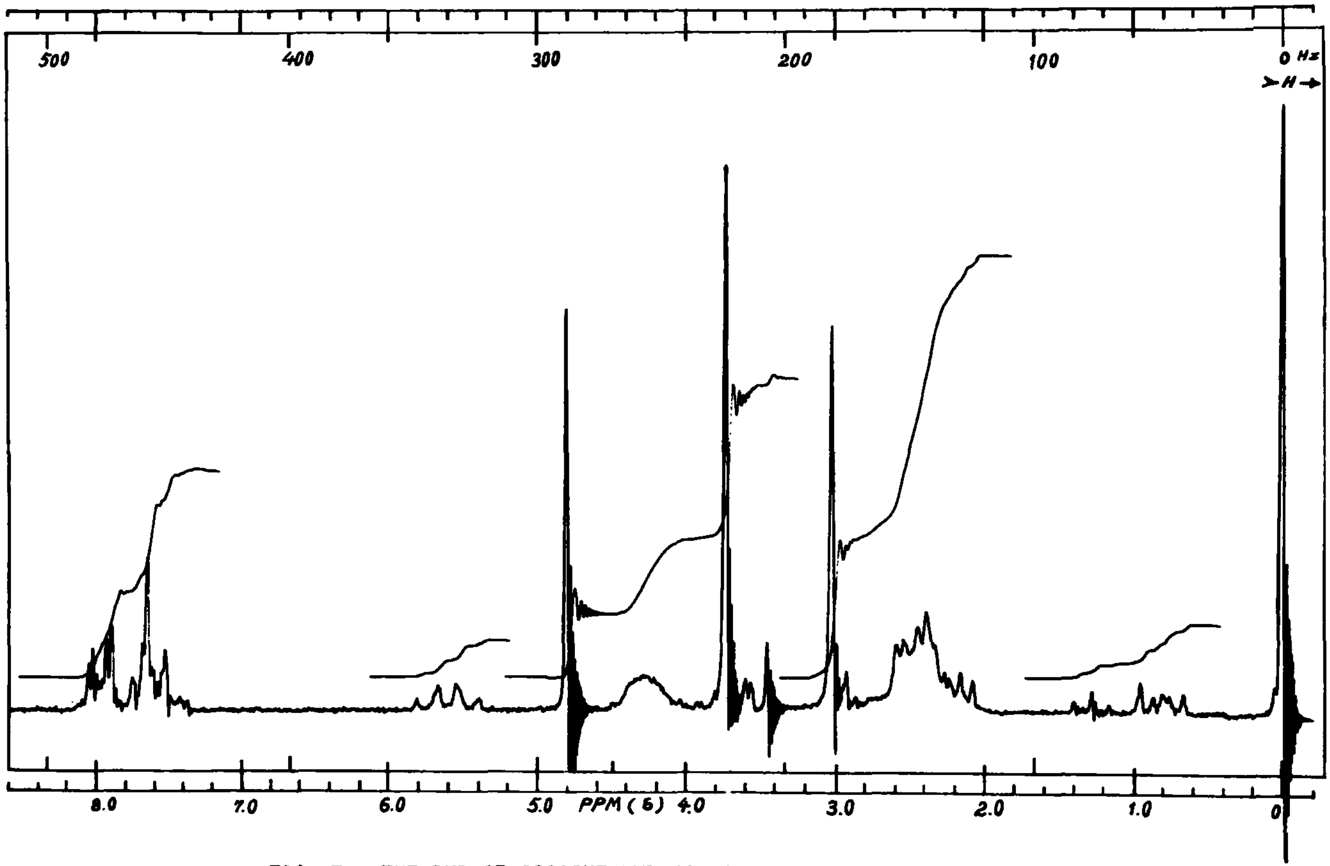


FIG. 3. THE PMR OF COCAINE HYDROCHLORIDE IN D_2O

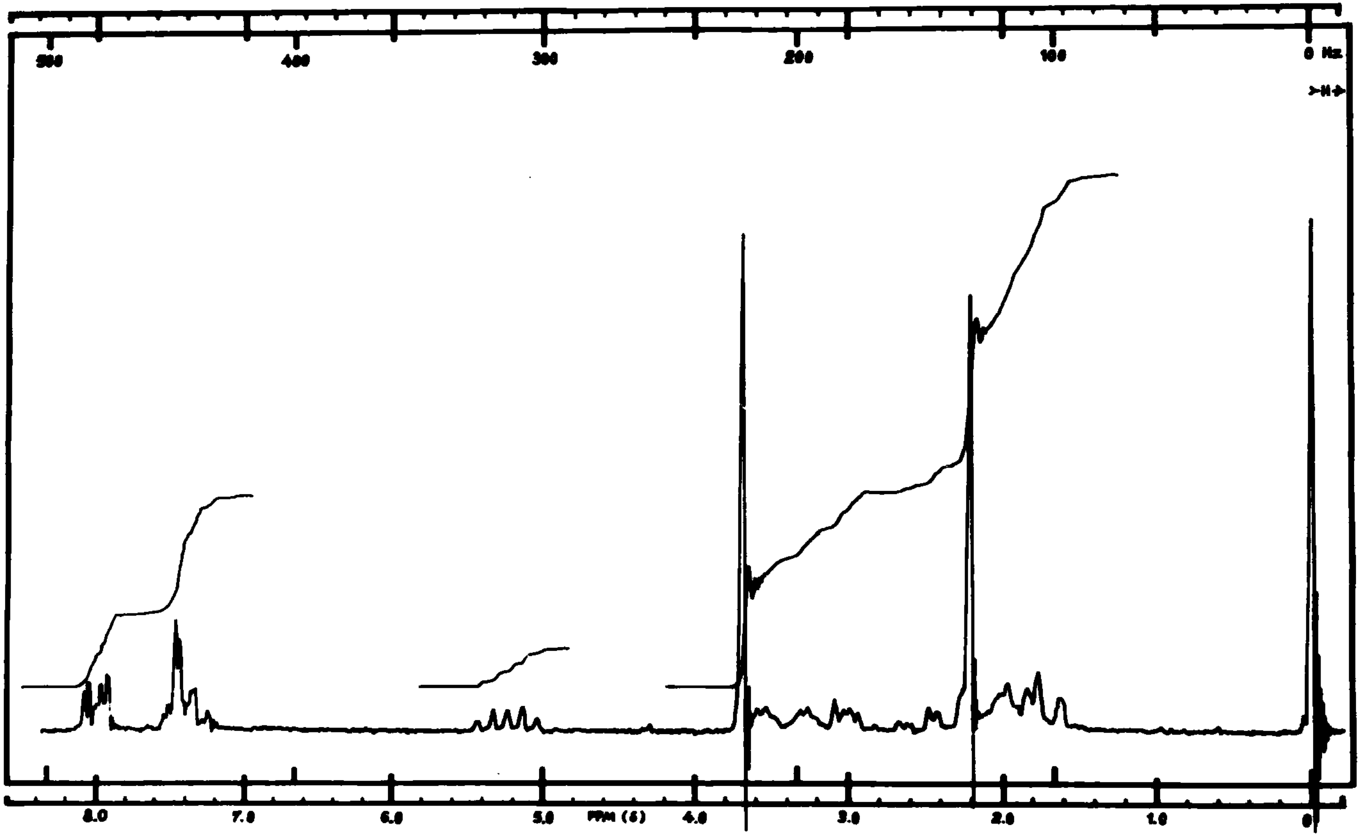


FIG. 4(A) THE PMR OF COCAINE IN $CDCl_3$

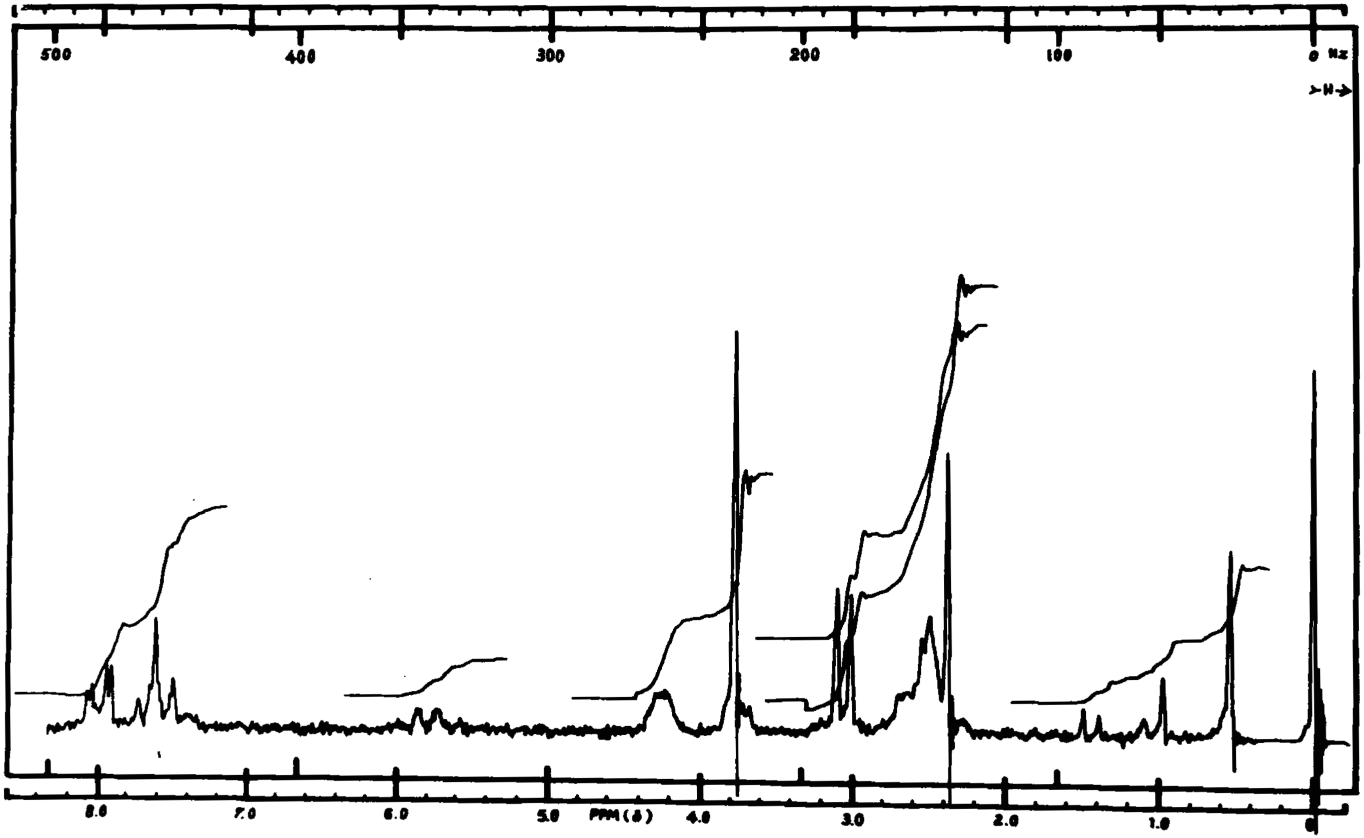


FIG. 4(B) THE PMR OF COCAINE IN TFA

Table 6. PMR Characteristics of Cocaine and Cocaine Hydrochloride.

Group	Chemical Shift (ppm)		
	Cocaine hydrochloride	Cocaine $CDCl_3$	Cocaine TFA
Aromatics	7.40-8.13 (m)	7.13-8.13 (m)	7.36-8.13 (m)
13, 17 HS	7.96 (m)	8.00 (m)	8.00 (m)
14, 15, 16 HS	7.60 (m)	7.40 (m)	7.56 (m)
3 H	5.60 (q)	5.23 (si)	5.78 (q)
1, 5 HS	4.26 (bs)	-	4.26 (bs)
$O-CH_3$	3.70 (s)	3.70 (s)	3.76 (s)
$N-CH_3$	3.00 (s)	2.23 (s)	3.03 (d)

s = singlet, d = doublet, m = multiplet, q = quartet, bs = broad singlet, si = sixtet.

Other PMR data for cocaine (3,18,20) and cocaine hydrochloride (21) have also been reported.

2.5.3.2 ^{13}C-NMR

The ^{13}C-NMR noise decoupled and off resonance spectra are presented in Fig. 5 and Fig. 6 respectively. Both were recorded over 4000 Hz range in D_2O and in Dioxan on a Varian FT 80 A-80 MHz spectrometer, using 10 mm. sample tube and dioxan as a reference standard at 22°.

The carbon chemical shifts are assigned on the bases of the additivity principals and off resonance splitting pattern (Table 7).

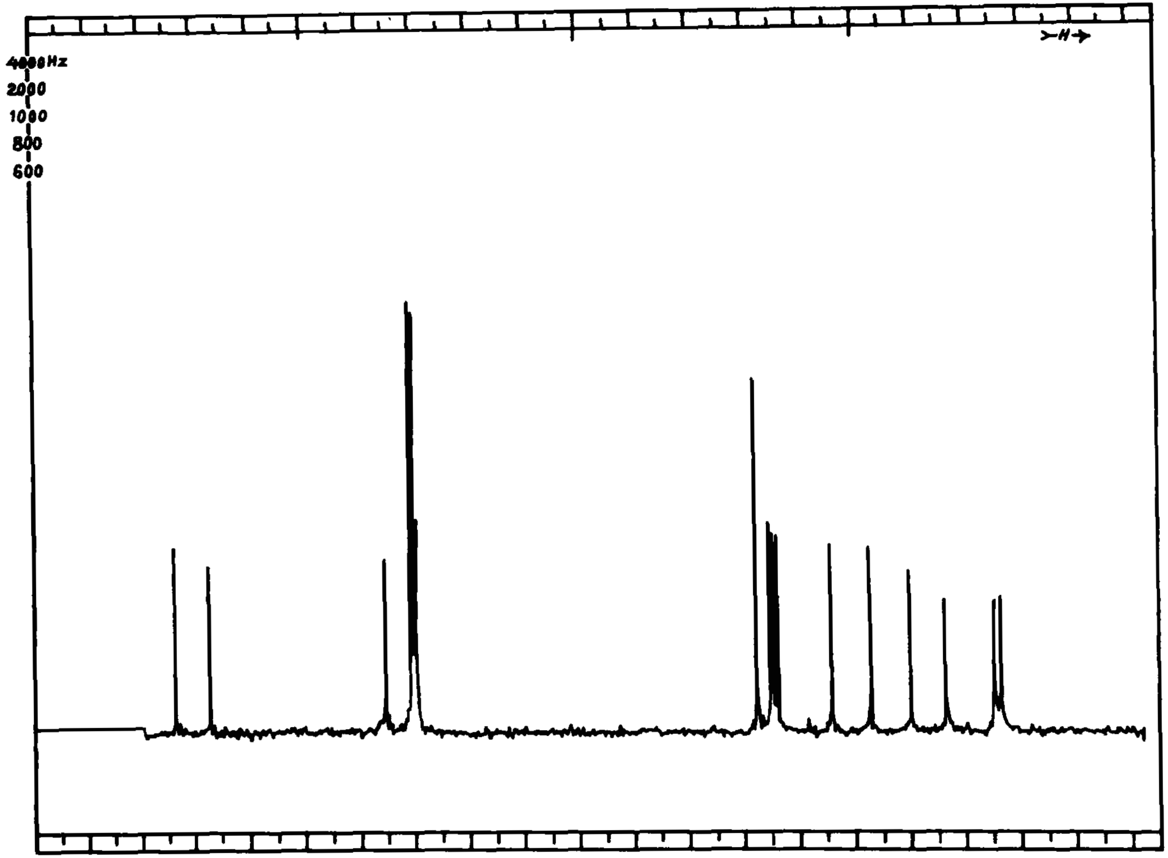


FIG. 5. THE ^{13}C-NMR NOISE DECOUPLED SPECTRUM OF COCAINE HYDROCHLORIDE

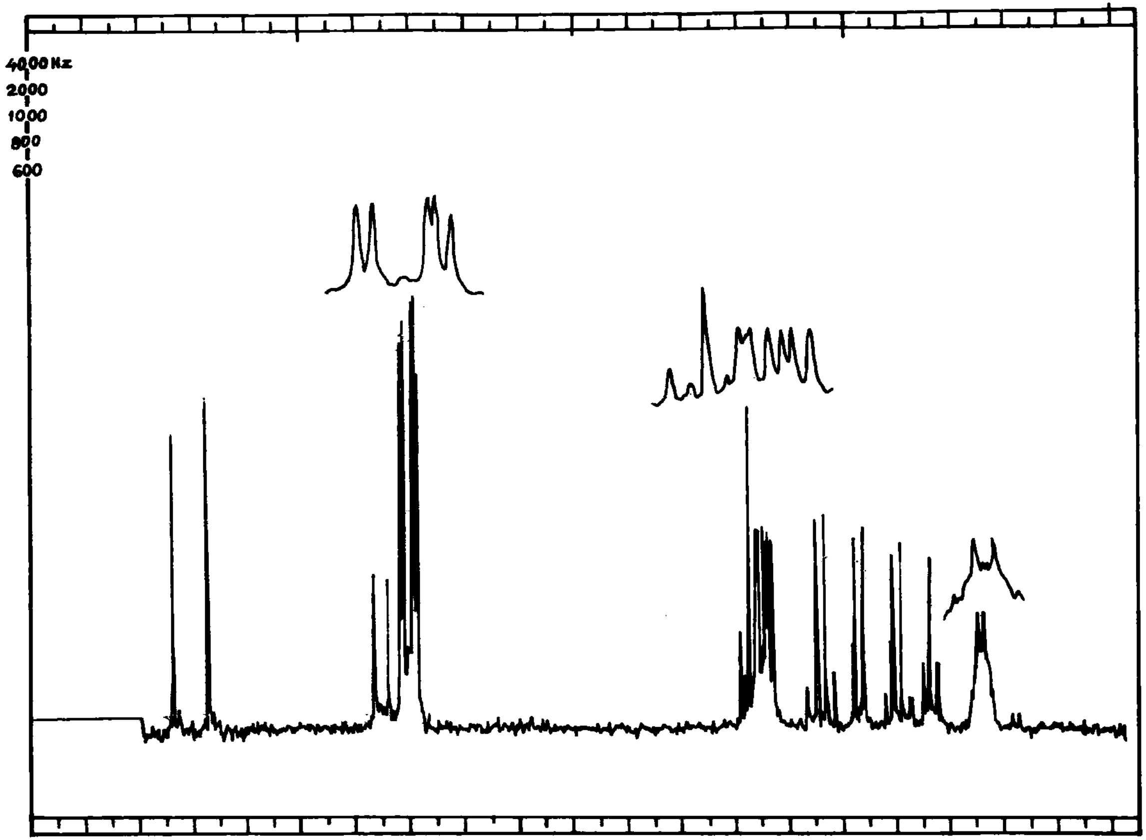


FIG. 6. THE ^{13}C-NMR OFF RESONANCE SPECTRUM OF COCAINE HYDROCHLORIDE

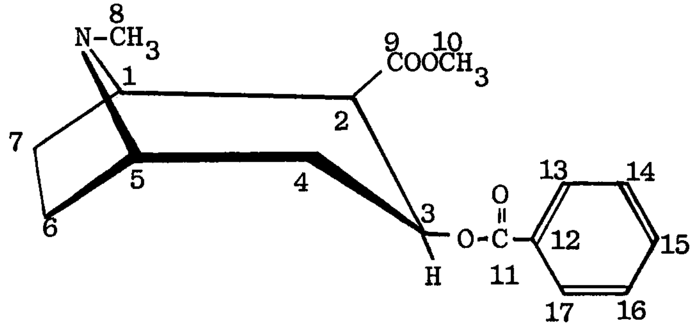


Table 7. Carbon Chemical Shifts of Cocaine HCl

Carbon no.	Chemical Shifts [ppm]	Carbon no.	Chemical Shifts [ppm]
C_9	173.51 (s)	C_1	63.79 (d)
C_{11}	167.09 (s)	C_{10}	54.05 (q)
C_{12}	135.06 (s)	C_2	46.98 (d)
C_{13}, C_{17}	130.18 (d)	C_8	39.85 (q)
C_{15}	129.69 (d)	C_4	33.41 (t)
C_{14}, C_{16}	129.22 (d)	C_7	24.59 (t)
C_3	67.39 (d)	C_6	23.45 (t)
C_5	65.05 (d)		

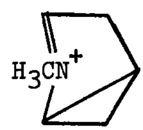
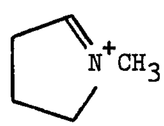
s = singlet, d = doublet, t = triplet, q = quartet

Other ^{13}C-NMR data for cocaine (20,22) have also been reported.

2.5.4 Mass Spectrum

The mass spectrum of cocaine hydrochloride is presented in Fig. 7 . This was obtained by electron impact ionization on a Varian MAT 1020 by direct inlet probe at 270°c. The electron energy was 70 eV. The spectrum scanned to mass 310 amu.
The spectrum (Fig. 7) shows a molecular ion peak M^+ at m/e 303 with a relative intensity of 15.60%. The base peak is 82 with a relative intensity 100%.
The most prominent fragments their relative intensities and some proposed ion fragments are given in table 8.

Table 8. Mass Fragments of Cocaine HCl

m/e	Relative intensity %	Ions
303	15.60	M^+ (cocaine)
272	6.75	-
198	11.37	-
183	9.26	-
182	84.84	183-H
122	9.85	-
105	29.97	-
97	10.03	-
96	24.06	
94	35.19	96-2H
83	35.83	

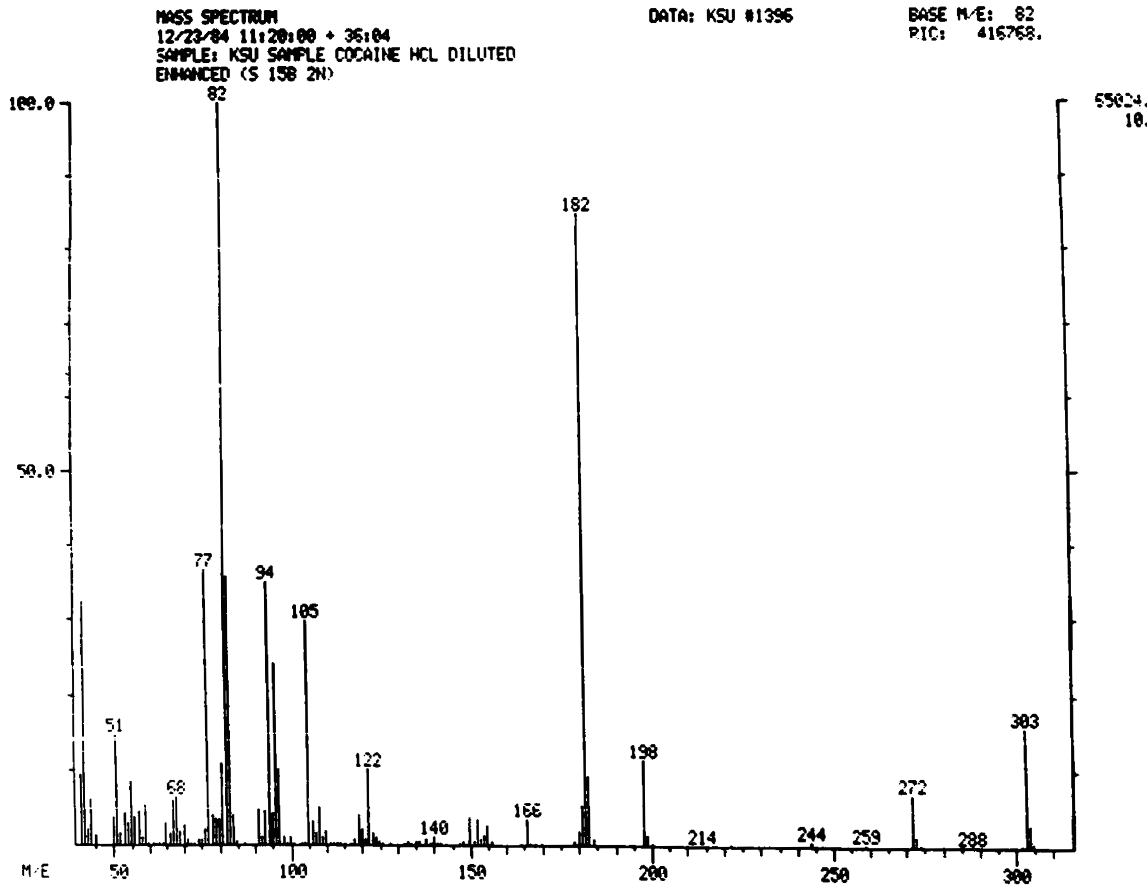
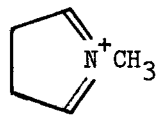


FIG. 7 : THE MASS SPECTRUM OF COCAINE HYDROCHLORIDE.

m/e	Relative intensity %	Ions
82	100.00	
81	10.63	82-H
77	36.66	-
68	6.39	-
55	8.29	-
51	14.54	-
42	32.43	$[CH_2{=}N{=}CH_2]^{+\cdot}$
41	9.45	42-H

Other reported mass spectra of cocaine has been also reported (23,24).

3. Isolation of Cocaine

Cocaine occurs in the leaves of *Erythroxylon coca* (Bolivian coca leaves), *Erythroxylon truxillense* (Peruvian and Javanese coca leaves) and other species of *Erythroxylon* family *Erythroxylaceae* (25,26).

Javanese leaves are usually the richest in total alkaloids, of which, the chief alkaloid is cinnamylcocaine, while the South American leaves contain less total alkaloids but higher percentage of cocaine (27,28).

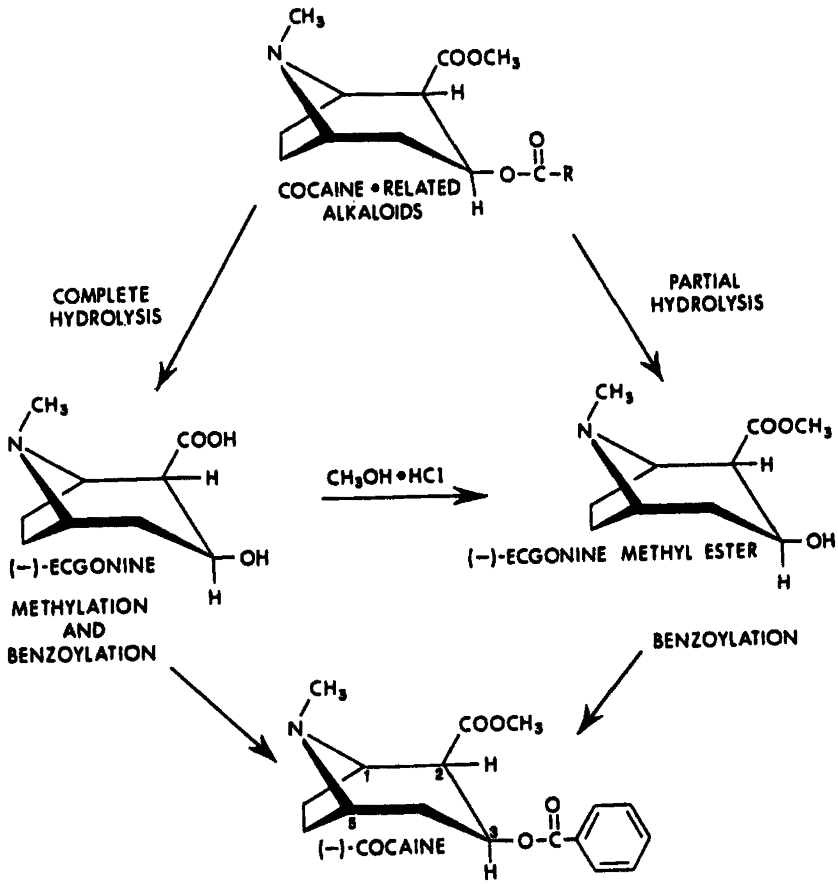
Java coca leaves are used to isolate cocaine commercially. The method depends upon isolating the total alkaloids including cocaine, hydrolyzing these alkaloids either to (-)-ecgonine or to ecgonine methyl ester, (-)-cocaine is then synthesized by methylation and benzoylation or by simple benzoylation (Fig. 8).

The Procedure

Powdered coca leaves are moistened with sodium carbonate solution and percolated till exhaustion with benzene.

- The benzene extract is shaken with dilute sulfuric acid. The collected acid extract is rendered alkaline with an excess of sodium carbonate. The resulting precipitated total alkaloids are extracted with ether.
- The ether extract is dried with anhydrous sodium sulfate, filtered and the ether is distilled off.
- The residue consisting of the total crude alkaloids is dissolved in methyl alcohol and the resulting solution is heated with sulfuric acid or with alcoholic hydrogen chloride (this treatment splits off any acids from ecgonine and simultaneously esterfies the carboxyl group to carboxymethyl group).
- After dilution, with water, the organic acids which have been liberated are removed with chloroform. The aqueous solution is then concentrated, neutralized and cooled with ice, whereupon methylecgonine sulfate crystallizes out and collected.
- This is now benzoylated by heating with benzoyl chloride or benzoic anhydride at about 150°C. Upon adding water and sodium hydroxide, cocaine is precipitated and extracted with ether.
- The ether extract is concentrated to crystallization, the crystallized cocaine is collected and recrystallized from

Fig. 8. The Commercial Preparation of Cocaine (27).



a mixture of acetone and benzene to give colorless prisms (Fig. 8).

Cocaine hydrochloride

This is prepared by adding cocaine to an alcoholic solution of hydrochloric acid and the resulting salt is purified by subsequent recrystallization.

4. Synthesis of Cocaine

4.1 Partial Synthesis

Cocaine can be synthesized by methylation and benzoylation of (-) ecgonine. Thus upon heating a mixture of (-)-ecgonine, benzoic anhydride and methyl iodide at 100°, (-)-cocaine is resulted (29).

(-)-Ecgonine is esterified with methanol to yield (-)-ecgonine methyl ester and this upon simple benzoylation with benzoyl chloride gives (-)-cocaine (30-32).

4.2 Total Synthesis

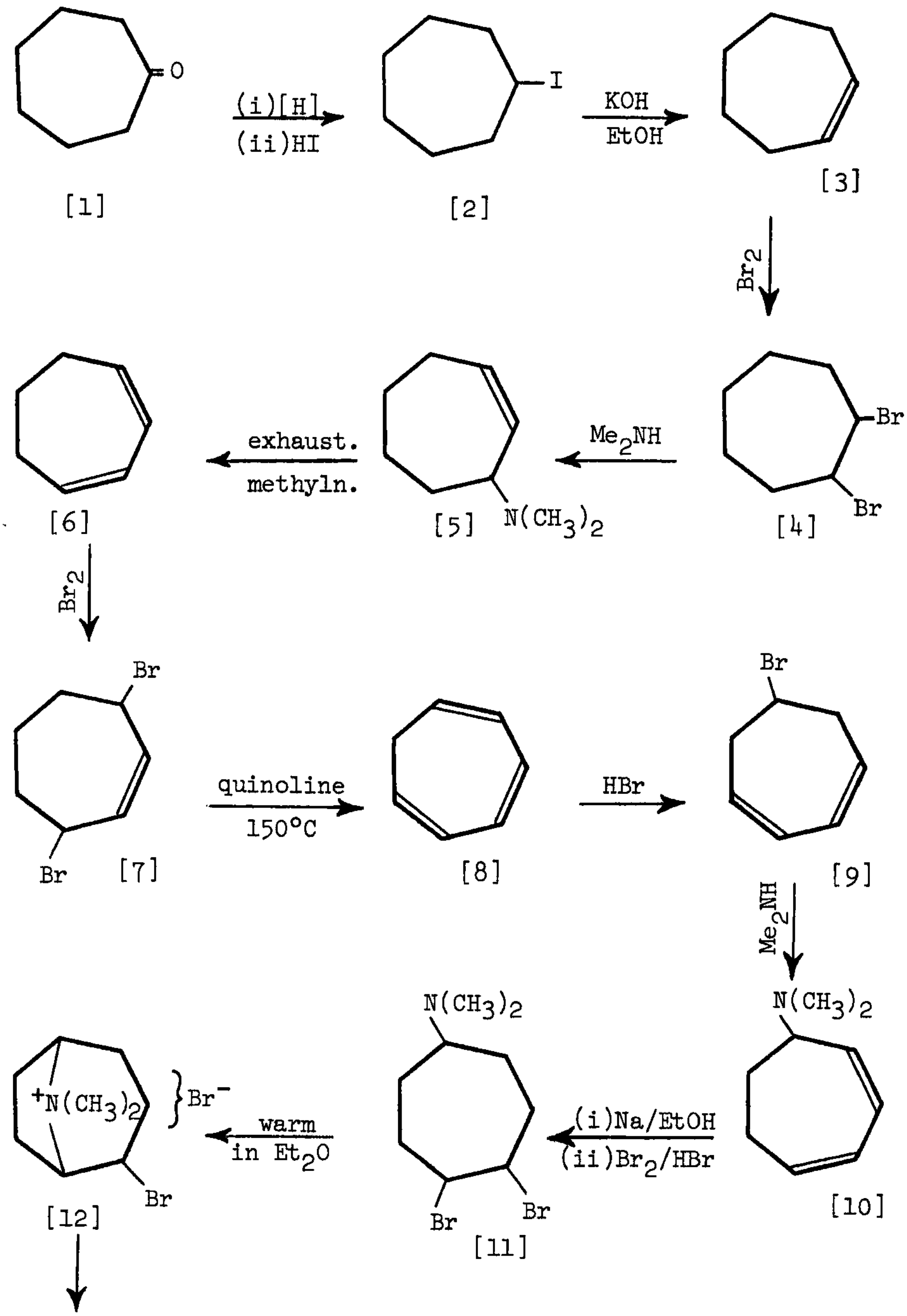
Since cocaine is an ester alkaloid consists of ecgonine methyl ester and benzoic acid, schemes for the total synthesis of both are required.

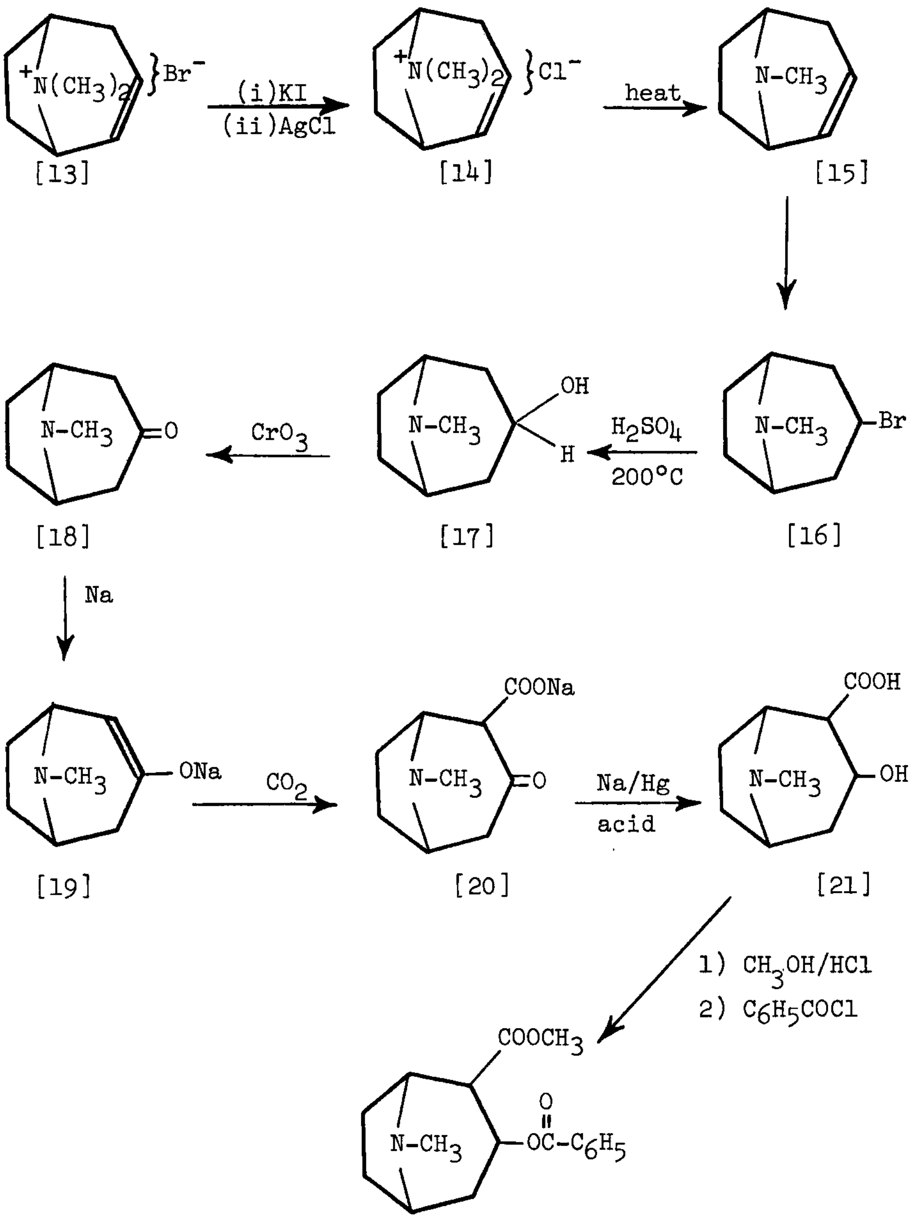
4.2.1 Total Synthesis of Ecgonine

Two schemes for the total synthesis of ecgonine are known.
Scheme I: Willstätter's total synthesis of ecgonine (1).

Suberone (cycloheptanone) [1] is reduced to suberol which is treated with hydrogen iodide to give suberyl iodide [2]. This is treated with potassium hydroxide in ethanol to give cycloheptene [3]. Cycloheptene is brominated to give 1,2-dibromocycloheptane [4] which is treated with dimethylamine to yield dimethylaminocyclohept-2-ene [5]. The latter is converted to cyclohepta-1,3-diene [6] by exhaustive methylation. [6] is brominated at 1,4-positions to give 1,4-dibromocyclohept-2-ene [7]. Elimination of two moles of the

SCHEME I: Willstätter's total synthesis of cocaine



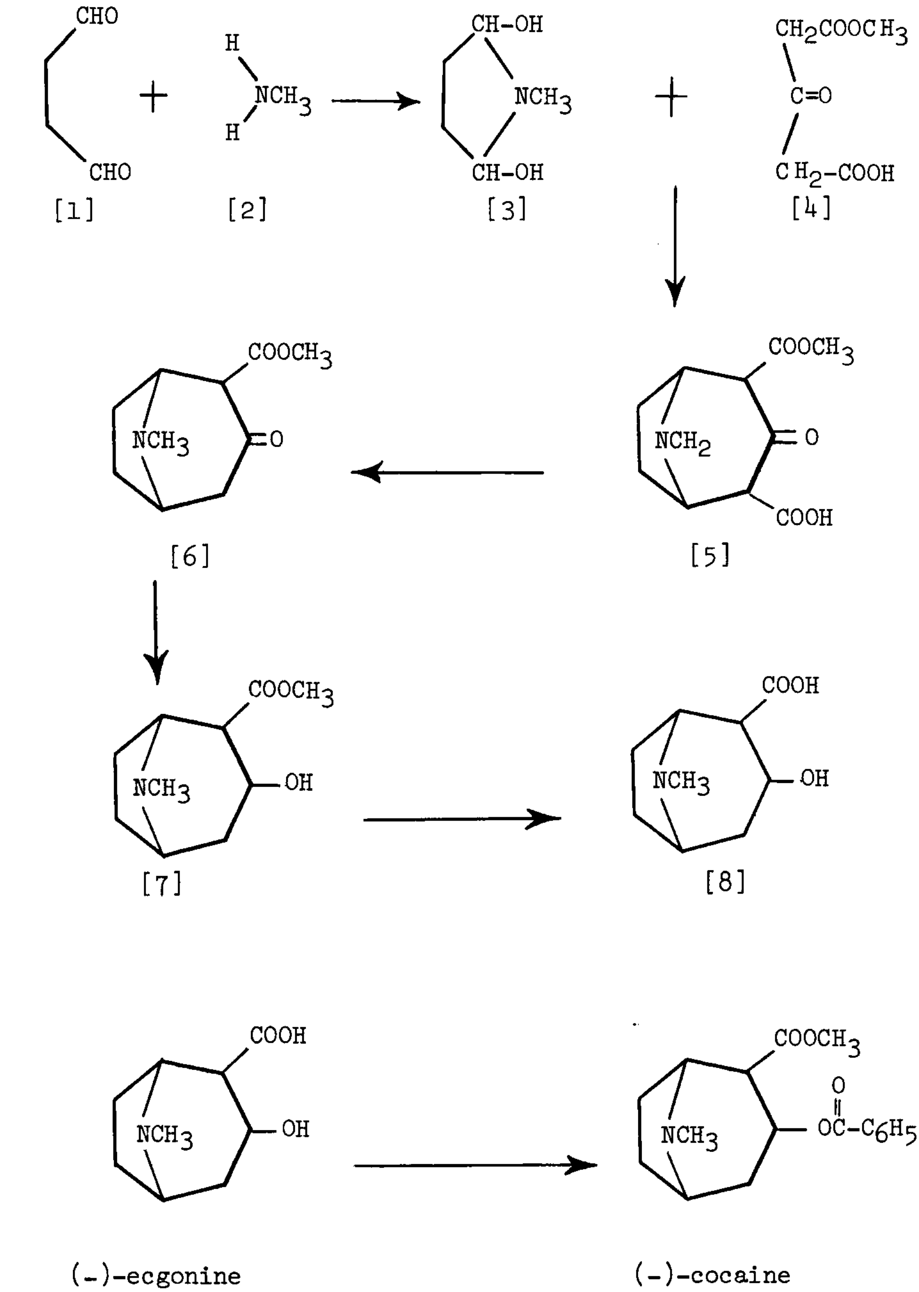


[13] [14] [15] [16] [17] [18] [19] [20] [21] [22]

hydrogen bromide of [7] is effected by quinoline to give cycloheptatriene [8]. Substance [8] is treated with hydrogen bromide to give bromocyclohepta-3,5-diene [9] which is reacted with dimethylamine to give dimethyl aminocyclohepta-2,4-diene [10]. The latter is treated with sodium in ethanol followed by bromination to give 1,2-dibromo-5-dimethylamino-cycloheptane [11]. This is warmed in ether when intramolecular alkylation occurs to give 2-bromotropane methobromide [12]. Hydrogen bromide is eliminated from [12] by the action of alkali to yield tropidine methobromide [13]. This is transformed to tropidine methochloride [14] by the action of potassium iodide followed by the action of silver chloride. Substance [14] is pyrolized to give tropidine [15]. Hydrogen bromide is added to an acetic acid solution of tropidine [15] to yield 3-bromotropane [16] which is hydrolysed with 10% sulfuric acid at 200-210° to give pseudotropine [17]. ψ-tropine [17] is oxidized with chromium trioxide to give tropinone [18]. This ketone upon treatment with sodium, (Kolbe-Schmitt type of reaction) gives the intermediate [19] which in the presence of carbon dioxide gives sodium tropinone carboxylate [20]. Upon reduction of [20] followed by acid treatment yields (±)-ecgonine [21]. (±)-Ecgonine [21] is resolved with (+)-tartaric acid to furnish (-)-ecgonine.

Scheme II: Total synthesis by adaptation of Robinson's tropinone synthesis (2). Succindialdehyde [1] is condensed with methylamine [2] to give biscarbinolamine [3]. This in turn condensed with acetondicarboxylic acid monomethyl ester [4] to give the condensate [5]. The latter is decarboxylated to yield ecgoninone methylester [6]. This β-ketoester is reduced with sodium amalgam to give ecgonine methyl ester [7], which is hydrolyzed to (±)-ecgonine [8]. [8] is resolved with (+)-tartaric to render (-)-ecgonine.

SCHEME II: Total synthesis by adaption of Robinson's method

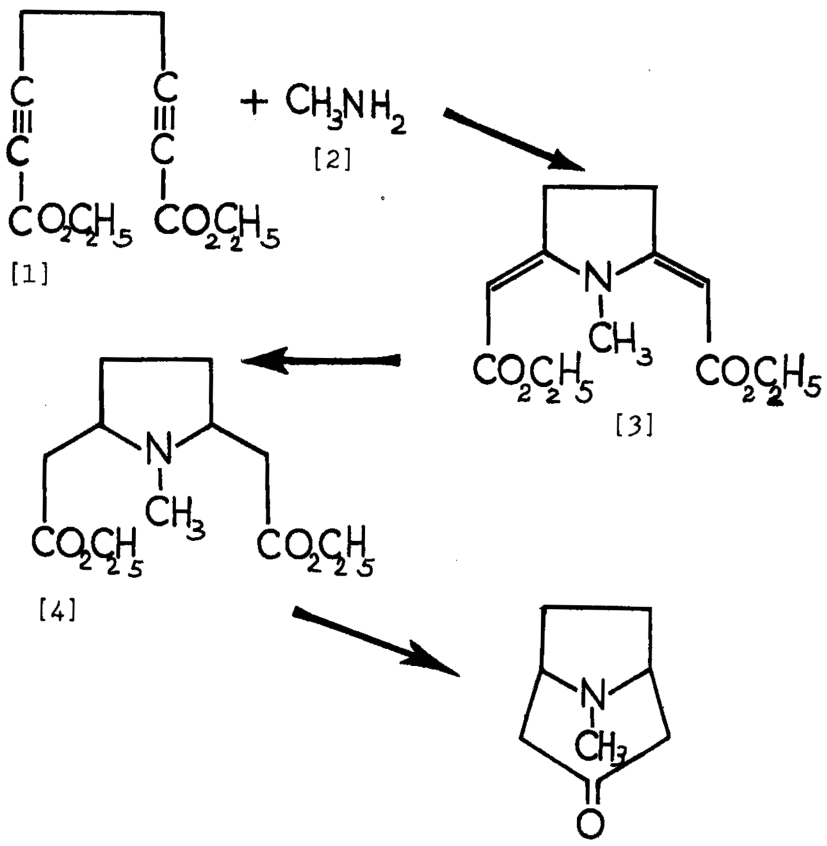


Synthesis of Cocaine

[-]-Ecgonine is esterified first with methanol and hydrochloric acid, then with benzoyl chloride to yield (-)-cocaine.

An alternate synthesis of tropinone has been reported (33). Tropinone can be converted into ecgonine by Willstätter's method (Scheme I).

Diyne diester [1] is condensed with methylamine [2] to give the pyrrolidine diester [3]. Catalytic hydrogenation gives [4] which on Dickmann cyclization, hydrolysis and decarboxylation yields tropinone [5].



4.2.2 Total Synthesis of Benzoic Acid

Benzoic acid is known acid and occurs in several sources in nature.
It can be prepared by heating gum benzoin when benzoic acid sublimes (34). It can also be prepared from hippuric acid which on boiling with mineral acids is hydrolysed to glycine and benzoic acid (34).
It is prepared exclusively from toluene as follows:-
Toluene is converted into benzotrichloride by treatment with chlorine and this is hydrolysed by lime water to calcium benzoate, from which benzoic acid is precipitated by the addition of hydrochloric acid and purified by recrystallization from water. Benzoic acid is prepared in large quantities by catalytic oxidation of toluene (34).

Benzoyl chloride required to prepare cocaine is prepared by warming benzoic acid with phosphorus pentachloride or preferably thionyl chloride or by the action of chlorine on benzaldehyde (34).

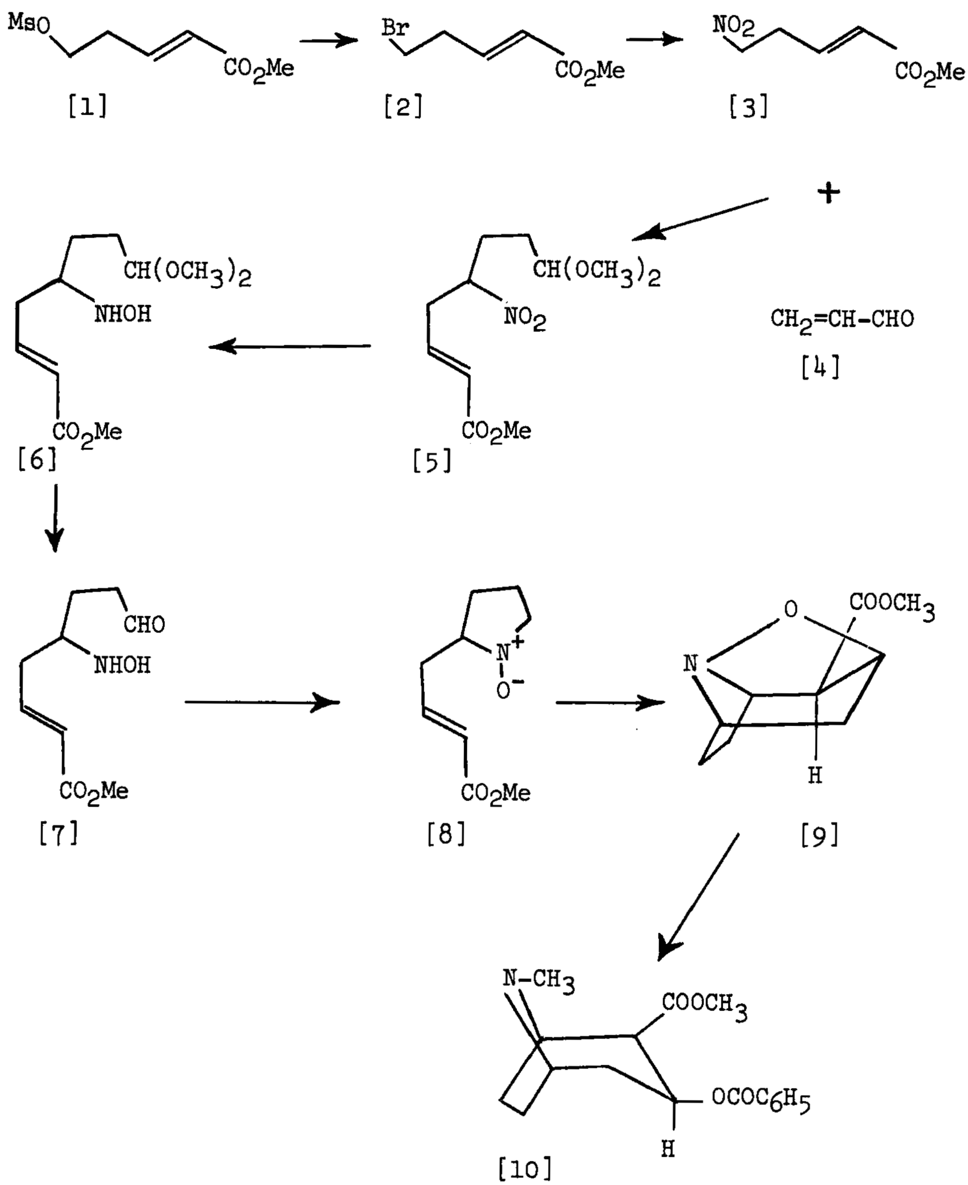
$$C_6H_5.\ COOH + SOCl_2 = C_6H_5.\ COCl + HCl + SO_2$$

$$C_6H_5.\ CHO + Cl_2 = C_6H_5.\ COCl + HCl.$$

4.3 Stereoselective Synthesis of dl-Cocaine

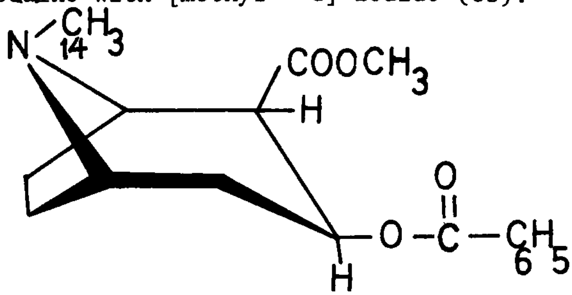
Stereoselective synthesis of dl-cocaine was described (3).
Mesylate olefin [1] is brominated to the bromo-ester [2] which is converted into the nitroester [3]. This is condensed with acrolein [4] in the presence of methanol containing sodium methoxide to give the dimethylacetal [5]. The nitrogroup of [5] is reduced with zinc and aqueous ammonium chloride solution to produce the hydroxylamine acetal [6] which upon acidification generates the hydroxylamine aldehyde [7]. [7] is cyclized to the nitron [8] which is converted into the cycloadduct [9].
The cycloadduct [9] is methylated with methyliodide in methylene chloride affords methiodide [9] which is treated with activated zinc in 50% aqueous acetic acid at 70°c in order to effect the scission of the nitrogen-oxygen bond to provide ecgonine methylester, which is then benzoylated to afford dl-cocaine [10].

Stereoselective Synthesis of dl-Cocaine

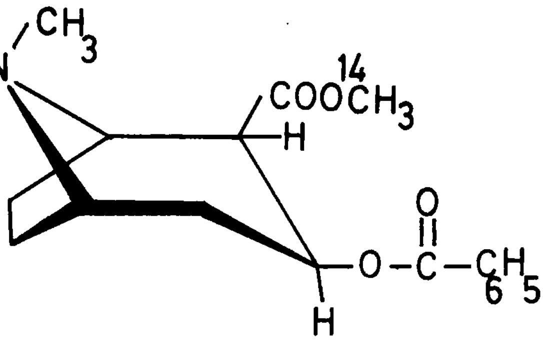


4.4 Synthesis of Radioactive Cocaines

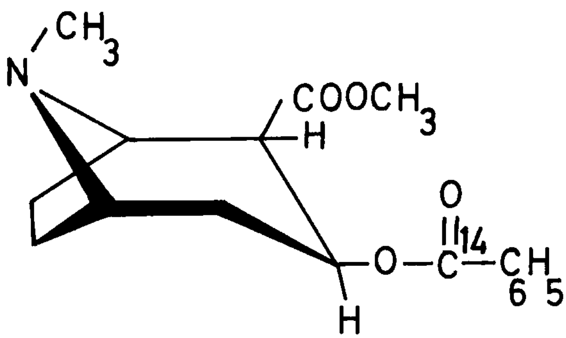
[N-methyl-^{14}C] cocaine is prepared by N-methylation of norcocaine with [methyl-^{14}C] iodide (35).



[Ester methyl-^{14}C] cocaine is prepared by methylation of benzoylecgonine with diazomethane-^{14}C (35).



[Benzoyl-α-^{14}C] cocaine is prepared by benzoylation of ecgonine methylester with benzoylchloride-α-^{14}C (35).



Methyl-tritiated derivatives of (-)-cocaine and (+)-ψ-cocaine were prepared (36).

5. Biosynthesis of Cocaine

It has been assumed that the tropine and ecgonine moieties of hyoscyamine and cocaine respectively arised from the aminoacid ornithine (37,38). The aromatic carboxylic acids of both alkaloids are built up from phenylalanine which is formed in plants from shikimic acid (38,39).

Using radioactive tracer technique, it was found that the administration of [3'-^{14}C] phenylalanine to *Erythroxylon novogranatense* yielded radioactive cocaine in which all the activity resided in the carboxyl group of the benzoic acid moiety of cocaine (40).

Feeding sodium [1-^{14}C] acetate and [methyl-^{14}C] methionine into the same species, resulted in the isolation of labelled cocaine (41).

It was also found that feeding sodium [1-C] acetate to *E. coca* plants, radioactive cocaine was isolated in which about 60% of the activity were located in the ester methyl group, 30% in the carboxyl group of the benzoic acid moiety and 8.7% in the ecgonine residue (42).

Leete (43) has fed the following radioactive precursor to *Erythroxylon coca* plants:-

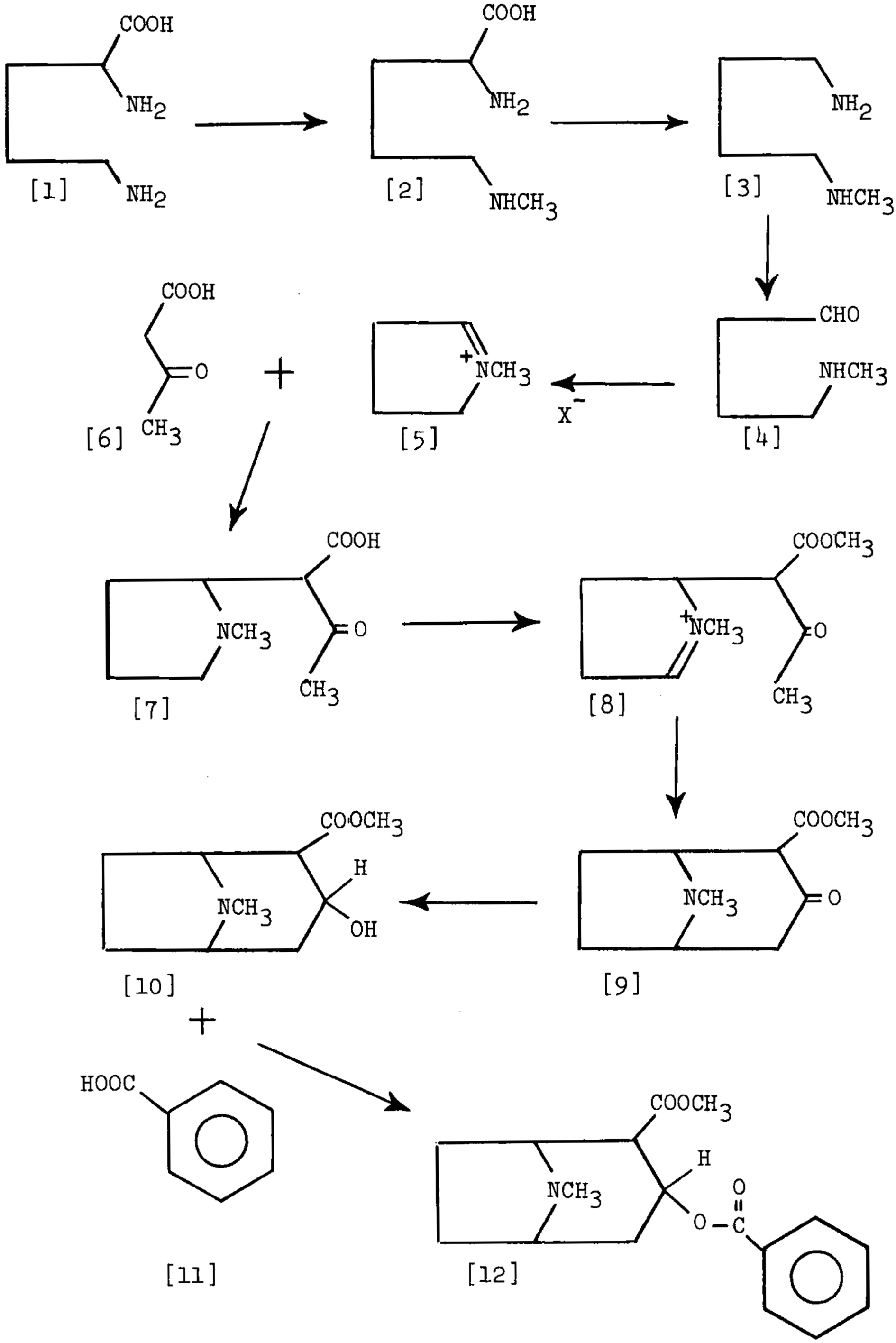
DL-[5-^{14}C] ornithine hydrochloride, DL-[2,3-^{13}C,5-^{14}C] ornithine hydrochloride, DL-[2-^{14}C] ornithine hydrochloride, [2-^{13}C, ^{14}C]-N-methyl-Δ^1-pyrrolinium acetate, [2-^{14}C] -N-methyl-Δ^1-pyrrolinium chloride, sodium [1-^{14}C] acetate and [carboxyl-^{14}C] nicotinic acid. Radioactive cocaine resulted from each of the above precursors with variable level of radioactivity.

Cocaine containing a significant level of radioactivity was obtained by painting the leaves of the *Erythroxylon coca* with an aqueous solution of DL-[5-^{14}C] ornithine hydrochloride (43). A systematic degradation of this cocaine indicated all the activity was located at the brigehead carbons (C_1 and C_5) of its tropane moiety and equally divided between these positions (43).

Leete therefore, proposed the following biosynthetic pathway of cocaine (43).

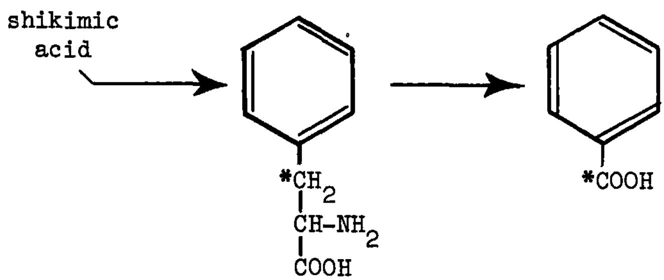
Ornithine [1] is incorporated into cocaine via δ-N-methylornithine [2] which is formed by N-methylation of [1]. [2] was isolated in radioactive form after feeding [5-^{14}C] or [5-^{3}H] ornithine to Atropa belladonna (44). Decarboxylation of [2] yields N-methylputrescine [3], an established precursor of the tropane nucleus of hyoscyamine and scopolamine (45-47). Oxidation of [3] affords 4-methylaminobutanal [4]. This is cyclized to give

Leete's Scheme: Biosynthesis of Cocaine



N-methyl-Δ^1-pyrrolinium salt [5] which is condensed with acetoacetate [6] to afford hygrine-l'-carboxylic acid [7]. [7] is dehydrogenated and esterified to give dehydrohygrine-l'-carboxymethylester [8]. The latter is cyclized to yield ecgoninone methylester [9]. Stereospecific reduction of [9] affords ecgonine methylester [10]. Ecgonine methylester [10] is finally esterified with benzoic acid [11] to give cocaine [12].

Benzoic acid is formed from the aminoacid phenylalanine (39) which is formed in plants from shikimic acid



6. Metabolism of Cocaine

Cocaine is well absorbed from all sites of applications including mucous membranes and the gastrointestinal mucosa (48-49). Absorption is enhanced in the presence of an inflammation (48,49).
After absorption, cocaine is degraded by plasma esterases (50) and in some animals by hepatic enzymes (51) to a number of metabolites (52-63). Some cocaine is excreted unchanged in the urine (51,52).
The major metabolites of cocaine in man and animals are benzoylecgonine, ecgonine, ecgonine methylester and norcocaine (52-57). Minor metabolites in man are ecgonine ethylester, cocaethylene, m-hydroxycocaine and ecgonidine methylester (56,57), these metabolites have been identified in multiple intoxication and overdoses of cocaine (57). Other metabolites which can be detected in animals are benzoylnorecgonine and norecgonine (58-60).
The metabolism of cocaine in man and animals is presented in Fig. 9, and the structures of cocaine and its metabolites are shown in Fig. 10 (52).

7. Pharmacokinetics

The pharmacokinetics of cocaine have been reported by several authors. Peak serum levels occur in 3-5 minutes following intravenous administration; in 20-60 minutes following intranasal administration and in 60-90 minutes after oral administration (64).
Plasma levels of cocaine 30 and 45 minutes post-administration of 1.5 mg/kg of a topical intranasal 10% cocaine solution, were 331 and 320 ng/ml respectively (65).
Following intranasal application of 1-5 mg/kg of cocaine to 13 surgical patients, plasma levels reached peak concentrations of 120-474 ng/ml at 15-60 minutes, and then decreased overthe next 3-5 hours (66).
Plasma level of cocaine after smoking one cigarette (75 mg of cocaine) in 3 minutes was 251 ng/ml with a range of 91-462 ng/ml (67).
The average plasma level of cocaine after smoking 3 cigarettes for 15 minutes was 478 ng/ml with a range of 226-684 ng/ml (67). The half-life of cocaine in the plasma after oral or nasal administration is approximately one hour (68). The mean half-life of cocaine for intravenous injections in four human subjects was 41.4±8.2 minutes and the range was 19 to 64 minutes (69).

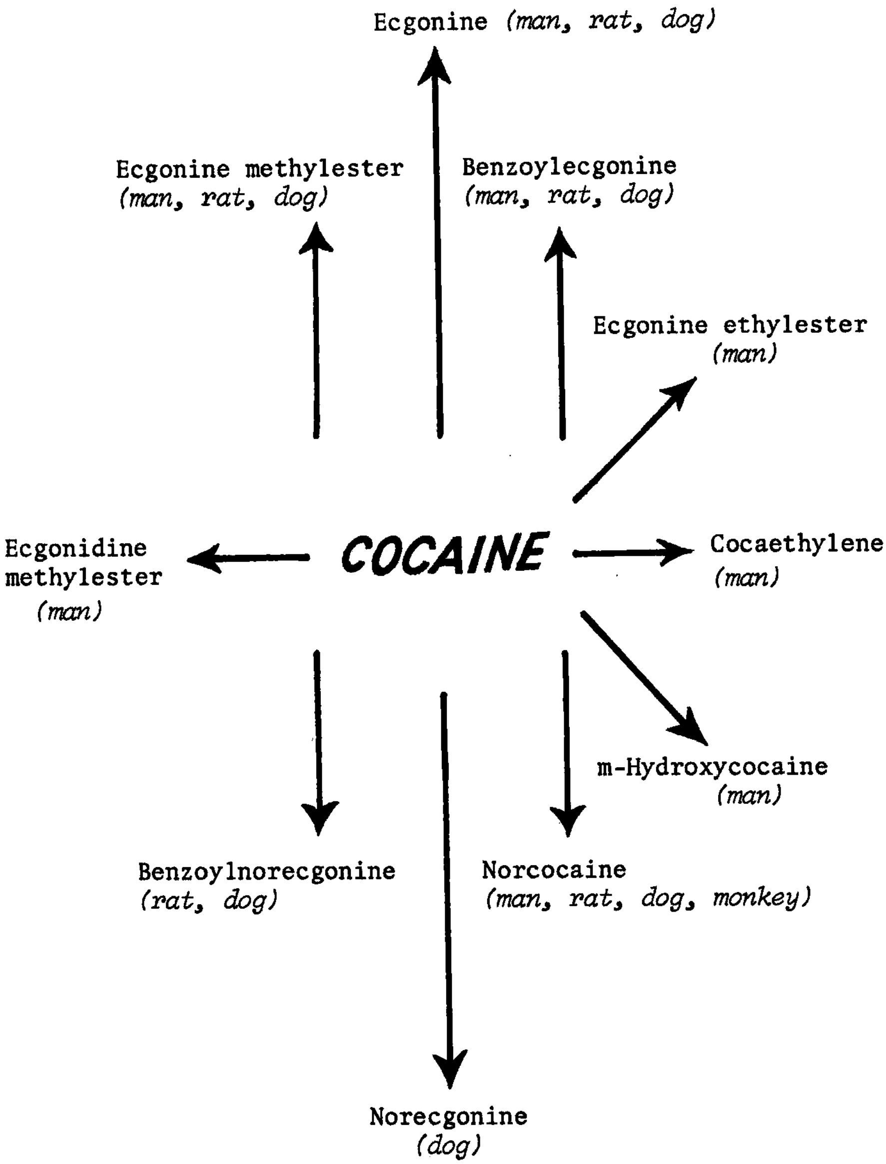
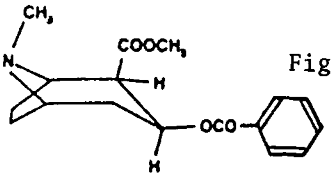
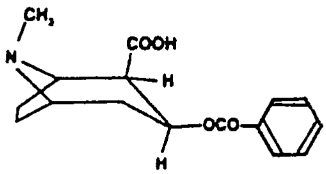


Fig. 9. The Metabolism of Cocaine (Cocaine Metabolites).

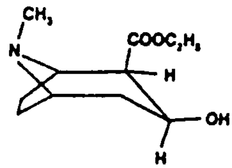
Fig.10. The Structures of Cocaine Metabolites.



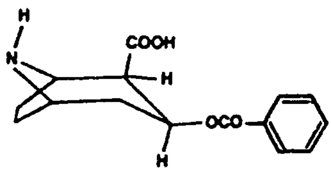
COCAINE



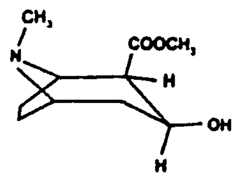
BENZOYLECGONINE



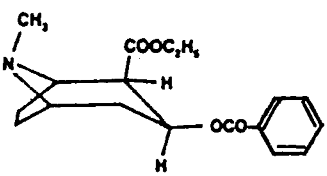
ECGONINE ETHYLESTER



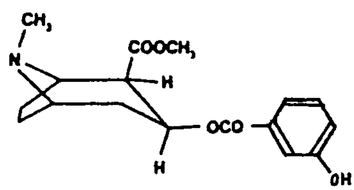
BENZOYLNORECGONINE



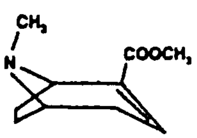
ECGONINE METHYLESTER



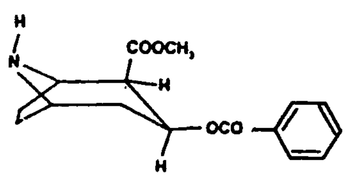
COCAETHYLENE



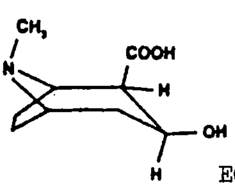
m-HYDROXYCOCAINE



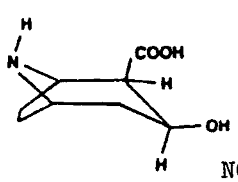
ECGONIDINE METHYLESTER



NORCOCAINE



ECGONINE



NORECGONINE

8. Drug Stability

Cocaine hydrochloride in the dry state, stored in a well closed container at room temperature, showed no decomposition after five years when examined by different physical and chemical data including spectrophotometrical evidence (70).

Solution of cocaine hydrochloride is liable to develope fungus growths and should contain a preservative. Stored in airtight containers, protected from light (49).

Aqueous solutions containing cocaine hydrochloride 5% and phenol 0.5% remained clear and colorless for a year at 0° to 4°, room temperature and 37°. A fall in pH from 4.6 to 3.9; 2.7 and 2.1 respectively, suggesting a chemical change, such solution should therefore be stored in a cool place (49).

Factors affecting the stability of cocaine in solutions were studied. No hydrolysis of cocaine was observed at pH values below 4.0, but hydrolysis was rapid when pH was greater than 5.5. Decreasing ionic strength also increased the rate of hydrolysis. With proper pH conditions, solutions of cocaine were stable at temperatures up to 24° for up to 45 days (71).

Brompton's cocktail containing morphine, cocaine and alcohol has a limited shelf life of about 3 weeks. This cocktail should be stored in well-closed light-resistant containers (72).

9. Methods of Analysis

9.1 Identification Tests

The following identification tests are mentioned under cocaine in the British Pharmacopoeia (14).

- The light absorption in the range 230 to 350 nm of a 2-cm layer of 0.001% w/v solution in 0.01 M hydrochloric acid exhibits a well defined maximum only at about 233 nm; with absorbance about 0.86.
- 0.1 g of cocaine is heated with 1 ml of sulfuric acid for five minutes at 100°, upon cooling and cautiously mixing with 2 ml of water; the aromatic odor of methyl benzoate is perceptible, and when the solution is cooled and allowed to stand for some hours, crystals of benzoic acid separate.
- 50 mg of cocaine is dissolved in 1.65 ml of 0.1 M hydrochloric acid, 8.5 ml of a 5% w/v solution of alum and 5 ml of potassium permanganate solution are added to the cocaine solution with stiring for several seconds; characteristic rectangular violet plates are formed.
- A saturated solution is alkaline to phenolphthalein solution.

The followings are identification tests mentioned under cocaine hydrochloride (14).

- To 0.5 ml of a 2% w/v solution, 0.5 ml of water and 0.1 ml of a 3% w/v solution of chromium trioxide are added; a yellow precipitate is formed which redissolves on shaking. Upon addition of the same reagent or hydrochloric acid, the precipitate reappears.
- 0.1 g of cocaine hydrochloride is heated with 1 ml of sulfuric acid for five minutes on a water bath, 2 ml of water are added carefully, methyl benzoate recognisable by its odor is produced. On cooling the solution, crystals are deposited.
- 50 mg are dissolved in 1.5 ml of water, 8.5 ml of a 5% w/v solution of alum and 5 ml of a 1% w/v solution of potassium permanganate are added and the resulting mixture is shaked for a few seconds; a crystalline precipitate is slowly formed, which when examined under a microscope, can be seen to consist of characteristic rectangular violet crystals.
- Specific optical rotation of a 2.5% w/v solution, -70° to -73°, calculated with reference to the undried substance.
- Yields the reactions characteristic of alkaloids and the reactions characteristic of chlorides mentioned in (14).

The following identification tests are mentioned under cocaine in the United States Pharmacopeia (73).

- The ultraviolet absorption spectrum of a 1 in 75,000 solution in dilute hydrochloric acid (1:120) exhibits maxima and minima at the same wavelengths as that of a similar solution of USP Cocaine Hydrochloride RS, concomitantly measured, and the respective molar absorptivities, calculated on the dried basis, at the wavelength of maximum absorbance at about 233 nm do not differ by more than 3.0%.
- It meets the requirements under identification - Organic Nitrogenous Bases (181), USP Cocaine Hydrochloride RS.
- 100 g are dissolved in a mixture of 0.4 ml of dilute hydrochloric acid (1 in 12) and water to make 5 ml, upon adding 5 drops of chromium trioxide solution (1:20); a yellow precipitate is formed, and it quickly redissolves when the mixture is shaken. Upon addition of 1 ml hydrochloric acid; a permanent orange-colored crystalline precipitate is formed.
- 10 mg are dissolved in 1 ml dilute hydrochloric acid (1:600) and evaporated on a steam bath just to dryness. The residue is dissolved in 2 drops of water, 1 ml of potassium permanganate solution (1 in 300) is added; a violet crystalline precipitate is formed, and it appears brownish violet when collected on a filter paper, and shows characteristic violet-red crystalline aggregates under the low power of a microscope, similar to those obtained from USP Cocaine Hydrochloride RS.
- 10 mg of the salt is dissolved in water (1:20), silver nitrate TS is added dropwise to the solution, a white precipitate is formed which is insoluble in nitric acid.

9.2 Microcrystal Tests

30 mg of cocaine hydrochloride dissolved in 25 ml of water. The following microcrystal tests were performed and microscopically examined.

- Mercuric chloride solution gives with cocaine after 15 minutes, cluster crystals which are shown in Fig. 11 (74).
- Marm's reagent gives with cocaine feathery needles which formed after 15 minutes, these are presented in Fig. 12 (74).
- A concentrated solution of cocaine hydrochloride (30 mg in 12.5 ml water) gives with potassium permanganate solution (1%), sharp jagged irregular blades

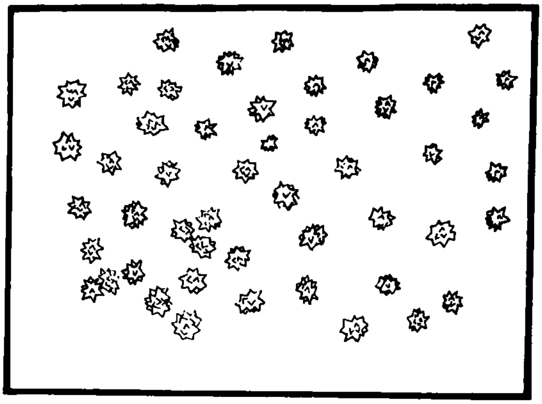


FIG. 11 : MICROCRYSTALS OF COCAINE WITH MERCURIC CHLORIDE.

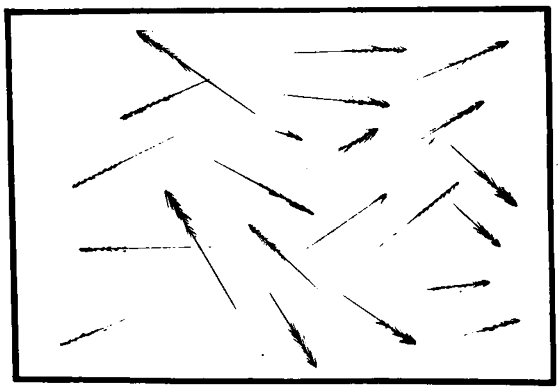


FIG. 12 : MICROCRYSTALS OF COCAINE WITH MARME'S REAGENT.

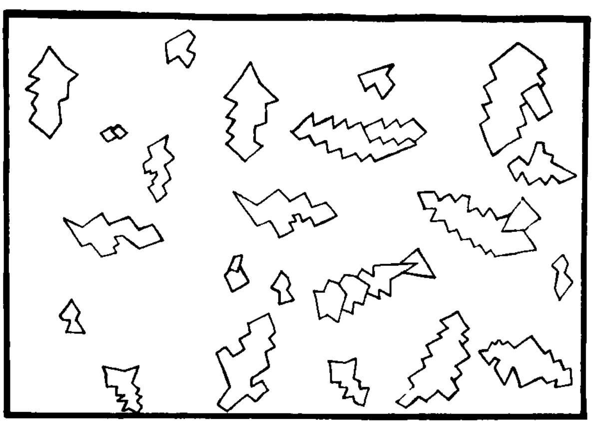


FIG. 13 : MICROCRYSTALS OF COCAINE WITH POT. PERMANGANATE.

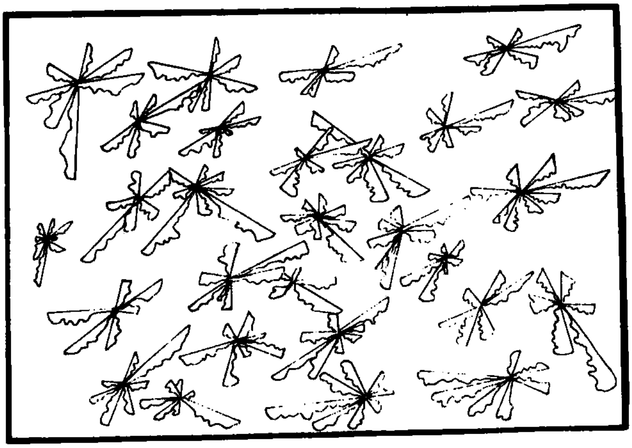


FIG. 14 : MICROCRYSTALS OF COCAINE WITH LEAD IODIDE.

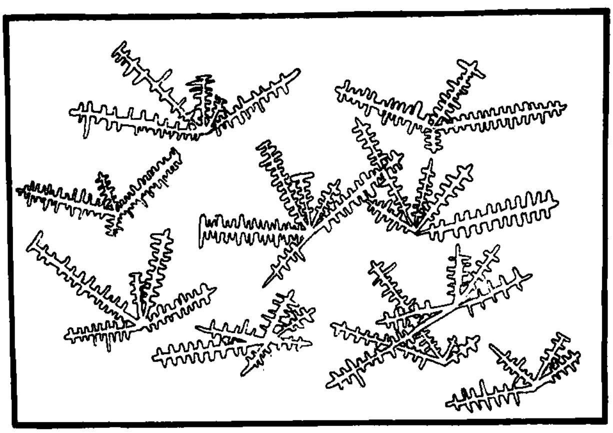


FIG. 15 : MICROCRYSTALS OF COCAINE WITH GOLD CHLORIDE.

after 7-10 minutes as in Fig. 13 (74).

- Lead iodide reagent yields with cocaine immediately, dentated irregular rosettes as presented in Fig. 14 (17,74).
- Gold chloride reagent yields with cocaine immediately, comb-shaped rosettes as shown in Fig. 15 (17,74).

The analytical methods of determination of cocaine and its metabolites in biological materials, have been reviewed by several authors (52, 75-78).

9.3 Titrimetric Methods

9.3.1 Non-aqueous Titration

British Pharmacopeia 1980 (14) and U.S. Pharmacopeia XX (73) described a non-aqueous titration for cocaine and its hydrochloride. The following are the procedures described in the US Pharmacopeia XX:-

Cocaine

Dissolve about 600 mg of cocaine, previously dried and accurately weighed, in 50 ml of glacial acetic acid, add 1 drop of crystal violet TS, and titrate with 0.1N perchloric acid VS to a green end-point. Perform a blank determination, and make any necessary correction. Each ml of 0.1N perchloric acid is equivalent to 30.34 mg of $C_{17}H_{21}NO_4$.

Cocaine hydrochloride

Dissolve about 500 mg of cocaine hydrochloride, accurately weighed, in a mixture of 40 ml of glacial acetic acid and 10 ml of mercuric acetate TS. Add 2 drops of quinalidine red TS, and titrate with 0.1N perchloric acid VS. Perform a blank determination, and make any necessary correction. Each ml of 0.1N perchloric acid is equivalent to 33.98 mg of $C_{17}H_{21}NO_4.HCl$.

9.3.2 Potentiometric Titration

Diamandis and Hadjiioannou (79) published a potentiometric method for the determination

of cocaine and some common alkaloids with a picrate-selective electrode. The method is based on formation of the insoluble picrates in ≃ 1 M concentration and were determined by direct potentiometry or by potentiometric titration with 8 mM- or 40 to 80 mM sodium picrate at pH 6, a picrate-selective electrode being used for indication. End-points were determined with use of Gran plots, for which the mean error was ± 2%.

9.4 Spectrophotometric Methods

9.4.1 Infra-red Spectrometry

Moss *et al* (80) had analysed the infra-red spectra of several drugs for similarities by techniques of numerical taxonomy including cocaine. Preliminary result for this set of drugs indicate that an expanded multi-variate approach to drug classification may be useful.

Moore (81) have described i.r. data for the identification of *cis* and *trans*- cinnamoyl cocaine in illicit cocaine seizures.

9.4.2 Ultraviolet

Kolosova (82) reported the use of ultraviolet spectrum for the detection of alkaloids (including cocaine) and barbiturates in objects of legal chemical examination. The drugs can be detected in biological materials by ultraviolet absorption in the region 200-400 nm.

Cis and *trans*- cinnamoyl cocaine in illicit seizures were identified (81). The isomeric alkaloids were found in the level of ≃ 1% in over half of the illict cocaine samples investigated. The author have presented the UV data as well as some other spectral data and g.l.c. conditions of analysis.

L-cocaine had been determined by circular dichroism (83). The samples are dispersed in water or methanol, and, after removal of

insoluble material, the principle absorption bands of L-cocaine are measured at 275 or 245 nm in a spectropolarimeter. The limit of detection is 10 uM (as calculated from the 245 nm band). In term of analysis time, the method compares favourably with other methods now in use; however, any DL-racemate will remain undetected. Results can be accepted as proof of the presence of L-cocaine in samples.

Toffoli and Avico (84) have reported chemical evaluation of crude cocaine and of the residue after industrial extraction of cocaine therefrom. The authors have described a procedure for the determination of cocaine in crude cocaine and another procedure for the determination of ecgonine and anhydroecgonine in the residue after extraction of cocaine. The procedure for determination of cocaine in crude cocaine is as follows:-

Dissolve 0.5 to 1 g of sample in 3% of H_2SO_4 (10 ml) at 0°. Still at 0°, add 6% potassium permanganate solution (8 ml) and 10% H_2SO_4 (1 ml at a time, and at 5 minutes intervals until the solution is decolorised. Set aside for 30 minutes, then add finely powdered oxalic acid until the precipitate formed has dissolved. Extract the resulting clear colorless solution twice with ether and discard the extracts. Make the solution alkaline with aqueous ammonia and extract four times with ether. Dry the combined extracts, evaporate and dry the residue for two hours at 60°, then for 12 hours over H_2SO_4 and weigh. Dissolve the residue in ethanol (20 ml) in the same container, add water (20 ml) and re-weigh. From the weight of solution, calculate the dilution (water) required) to give a density of 0.926 at 20°, i.e., 54% (v/v) of ethanol content. Measure the optical rotation and hence calculate the amount of cocaine. Dilute an aliquot of the solution 1:1000 with 0.1N HCl and measure the extinction at 274 nm; for cocaine in 0.1N HCl, $E^{1\%}_{1cm} = 38.2 \quad 2.0$. Two maxima are in fact

observed (at 274 and 233 nm), with molecular excitation coefficient of 1130 and 13,500, respectively. The direct weighing method the most reliable of these methods. Chromatography and electrophoresis of the sample before and after oxidation with potassium permanganate shows that only cocaine remains after this treatment.

Spectrophotometric assay of cocaine and some narcotics and alkaloids in galenical compositions have been reported (85). To assay cocaine in aqueous solution of its salt, the extinction of the diluted sample is determined at the wavelength for maximum absorption (257 to 286 nm) and compared with that of progressively diluted samples of stock solutions. The method is chiefly designed for use on aqueous preparations (e.g. from ampoules).

9.4.3 Fluorescence Analysis

Shih-Chung Tu (86) have reported fluorescein chloride spot test for the determination of cocaine and some othe r drugs containing amino and heterocyclic nitrogen. The determination is based on the formation of thodamine dyes. One mg fluorescein chloride and 1 drop of the drug (0.01-0.1%) were evaporated to dryness, fused with one drop of $ZnCl_2$; the melt was dissolved in 2 drops 10% alcoholic HCl solution. The fluorescence was determined with ultraviolet light; the color depends on the molecular structure. The test (2-4 y) was 2-5 times more sensitive than the Feigel test-tube method.

9.4.4 Phosphorimetry

Harbaugh et al (87) have reported qualitative and quantitative analysis of cocaine. The phosphorescence emission spectra, life times, and relative signals (peak emissions) of cocaine and some other drugs were determined with use of the apparatus and procedures previously described (88). For a multicomponent mixture, the parameters cited indicate which

of the drugs can be separated spectrally or temporarally or by a combination of the two techniques. Some mixtures, however, cannot be separated. Examples are given for the determination of the components of several synthetic binary mixtures of the drugs. In some instances where the emission spectra severely overlap. Time-resolved phosphorimetry provides a useful means for identifying these drugs even in some of their mixtures.

External heavy-atom effect on detection limits and life times of phosphoresence in time-resolved laser-excited phosphorimetry have been reported by Boutilier and Winefordner (89). In the instrument described, radiation from a pulsed nitrogen or flash lamp-pumped dye laser was directed via a filter to the sample contained in a quartz cell (30 cm X 2 mm i.d.) mounted in an n.m.r. spinner assembly. The emitted radiation was examined in a system incorporating a monochromator, photomultiplier, gated amplifier, signal generator, boxcar integrator, chart recorder, oscilloscope, signal overager, tape punch and minicomputer. Phosphoresence life-time and detection limits were determined at 77 K for solutions of phenanthrene and of several drugs (including cocaine) in ethanol-H_2O (1:9). In the presence of Ag^+, detection limits were often improved.

9.4.5 Colorimetry

Tomasch and Majer (90) have estimated some pharmaceutical esters which are used in pharmacy. The method of Hill (91) was modified and adopted for the determination of the esters. Five ml of the ethanolic solution of the ester (1 mg/ml) is added to 1 ml of 10% hydroxylamine hydrochloride in 80% ethanol, 2 ml of 10% sodium hydroxide in 90% ethanol and 5 ml peroxide-free ether; the mixture is shaken well, after 20 minutes treated with 2 ml HCl diluted 1:3 and 1 ml of 10% of ferric chloride ($FeCl_3$) in 0.1N HCl, diluted to 25 ml, filtered from sodium chloride and detected colorimetrically at 320 nm.

The use of cobalt thiocyanate in the colorimetric analysis of cocaine and some other organic basis have been reported (92). The authors have discussed earlier unpublished work by other authors on the precipitation and separation of alkaloids and organic bases with thiocyanates some metals and with cobalt in particular. The precipitation of such compounds with $CO(SCN)_2$ from aqueous medium results in two types of complexes, vis, a blue type (the more common) which the cobalt forms a complex anion with SCN^-, and a red, brown or violet type in which the cobalt forms a complex cation with organic base. Condition for the determination of various quaternary ammonium compounds and of cocaine in non-aqueous medium by extraction with benzene, chloroform or dichloromethane are discussed; a suitable reagent is prepared by dissolving $CO(SCN)_2$ in methanolic HSCN (obtained by cation exchange) to form a solution of $H_2CO(SCN)_2$. This is diluted with chloroform or dichloromethane to give 0.2N solution, which is added to a solution of the base in the same solvent. The extinction of the blue solution is measured at 625 nm.

Other colorimetric methods have been reported (93-95).

9.4.6 Mass Spectrometry

The mass analysed ion-kinetic-energy spectrometer was applied to the determination of cocaine in coca leaves and urine by use of single or multiple-ion monitoring technique (96); sensitivity was maximized by use of single-ion monitoring (with isobutane as reagent gas). The detection limit for cocaine in 1 μg of coca leaf being <1 ng. Multiple-reaction monitoring provided similar sensitivity to, and was more selective than single-ion monitoring. The effect of the method of sample introduction was important. For quantitative analysis, isotope dilution with analyte labelled with stable isotope was preferred; alternatively, standard-addition, external-

standard or calibration graph methods could be used. The graph (based on single-reaction monitoring) on peak area vs amount for solution of cocaine in methanol was rectilinear for 1 to 150 ng. Single reaction monitoring permitted detection of cocaine in unreacted samples (containing large quantities of many other drugs) and of benzoylecgonine in urine.

Cooks et al (97) have reviewed mass analyzed ion kinetic energy spectrometry (MIKE) and presented its application to the direct analysis of cocaine and cinnamoylcocaine in coca plant (Erythroxylum coca) tissues.

Moore (81) have presented m.s. data for the identification of cis and trans- cinnamoylcocaine in illicit cocaine seizures. The n.m.r. data and some other spectral data have also been presented.

9.5 Counter-Current Extraction

The separation of the mixtures of organic substances by a simplified method of counter-current extraction had been reported (98). The optimal conditions were found for the separation of two component alkaloid and other organic compound mixtures by the method of counter-current extraction in funnels. The dependance of the extraction with chloroform for quantitative separation of cocaine, and other alkaloids, on the pH values of the buffer solution is presented.

9.6 Chromatographic Methods

9.6.1 Thin Layer Chromatography

Clarke (17) described the following system:

Glass plates, 20 X 20 cm, coated with a slurry consisting of 30 gm of silica gel G in 60 ml of water to give a layer 0.25 mm thick and dried at 110° for 1 hour. A sample 1.0 µl of a 1% solution in 2N acetic acid, taken by a micro drop, is used. The solvent system consists of strong ammonia solution : methanol (1.5 : 100). It should be changed after two runs. Solvent is allowed to stand in the tank

for one hour. The assending chromatogram is developed in a tank 21 X 21 X 10 cm, the end of the tank being covered with filter paper to assest evaporation. Time of run 30 minutes. The location reagent is an acidified iodoplatinate spray, and the Rf value is 0.60.

Several solvent systems have been used for thin-layer chromatographic separation of cocaine and are shown in Table (9).

Table (9) Thin-Layer Chromatography of Cocaine

Absorbent and Solvent Systems	Visualizing agent	Rf	Ref.
Silica gel F 1500 LS 254 activated at 110° for 2 hours methanol : aqueous ammonia (100:1.5)	UV at 230 nm after extraction into chloroform	0.73	102
ethanol : chloroform (1:1)	UV at 230 nm after extraction into chloroform.	0.31	102
Silica gel 60 F_{254} 0.25 mm			
a) Chloroform : methanol conc. ammonia (9:10:1)	Sprayed by 5% H_2SO_4 followed by iodoplatinate	0.90	103
b) Ethyl acetate : methanol : water : conc. ammonia (85:10:3:1)	Sprayed by 5% H_2SO_4 followed by iodoplatinate.	0.87	103
Activated precoated silica gel plates of 0.25 mm thickness (Merck) Ethylacetate : n propanol: 28% ammonium hydroxide (40:30:3)	Acidic iodoplatinate (AIPA)	0.79	105
Precoated silica gel G, 26 X 20 cm, 0.25 mm layer *ethyl acetate : methanol 17:2 and 20 ml of 50% ammonia in a beaker.	Dragendoff then iodoplatinate	0.73	

Continued Table (9)

Absorbent and Solvent Systems	Visualizing agent	Rf	Ref.
Chloroform : methanol (1:1) and 20 ml of 50% ammonia in a beaker. The above two system are placed in the center of a tank.		0.73	110

Other thin layer chromatography systems have also been reported (17, 99-101, 104, 106-109, 111-119).

9.6.2 Paper Chromatography

Clarke (17) described the following system:

Whatman No. 1, sheet 14 X 6 inch, is buffered by dipping in a 5% solution of sodium dihydrogen citrate, blotting, and drying at 25° for one hour. It can be stored indeninitely. A sample of 2.5 μl of a 1% solution; in 2N acetic acid if possible, otherwise in 2N hydrochloric acid, 2N sodium hydroxide, or ethanol solvent, 4.8 g of citric acid in a mixture of 130 ml of water and 870 ml of n-butanol. (This solvent may be used for several weeks if water is added from time to time to keep the specific gravity at 0.843 to 0.844). The chromatogram is ascending, in a tank 8 X 11 X 15½ inch, 4 sheets being run at a time. Time of run, five hours, Rf value 0.38, location is done under ultraviolet light, strong absorption and the location reagent is iodoplatinate spray; strong reaction.

Bastos _et al_ (120) reported a method for routine identification of cocaine metabolites in human urine. The sample (10 ml) was adjusted to pH 8 to 9 with $NaHCO_3$ and extracted

chloroform : ethanol (3:2) (5 ml); the lower layer (containing, e.g. unmetabolized cocaine) was rejected. The upper layer was saturated with K_2CO_3 and centrifuged, and the pH of the ethanolic phase was adjusted to < 7 with dil. HCl. The extracted metabolites were converted into their butyl derivatives with butanol-H_2SO_4 and the solution was washed with toluene. The solution was then saturated with $NaHCO_3$ and the derivatives were extracted with cyclohexane (10 ml). The extract was evaporated, and the residue was dissolved in chloroform and applied to a polygram silica gel MN sheet. The chromatogram was developed with ethyl acetate-methanol-water (7:2:1), then for a short distance in the same direction with chloroform-acetone-aqueous ammonia(5:94:1). Alternatively, these two solvents were used for two-directional separation. Spots were detected by spraying with iodoplatinate reagent. El-Darawy and Mobarak (121) studied the chromatographic separation of some alkaloids including cocaine, on carboxymethylcellulose cation-exchange paper. From 5 to 8 µl of methanolic solution (1 mg per ml) of each alkaloid were applied to the paper and after development of the chromatogram, the spots were detected by viewing under 254 nm radiation or by spraying with Dragendorff reagent.

9.6.3 Gas Chromatography

Clarke (17) described the following system:

Column: 5% SE-30 on 60-80 mesh chromosorb W AW. 5 ft X 1/8 inch internal diameter stainless steel column. Column temperature: 230°, Carrier gas: Nitrogen gas flow: 30.7 ml per minute, Detector: flame ionisation, hydrogen 22 ml per minute. Retention time is 0.57 relative to codeine.

Several reports had been published concerning the chromatographic identification and separation of cocaine and its metabolites as shown in Table 10.

Table (10) <u>Gas Chromatography of Cocaine</u>

Column	Carrier gas	Detector	Remarks	Ref.
U-shaped column (6 ft X 0.25 in.) 2.5% pf SE-30 on Gas Chrom Q (100-120 mesh)	Nitrogen 25 ml/min. operated at 200°.	Flame ionisation	- The benzoyl ecgonine (a cocaine metabolite) is the detected substance which was obtained from urine. - The metabolite is separated by TLC then was subjected to GLC after being converted into its methyl derivatives by treatment with 1,1-dimethoxytrimethylamine at 70° to 80° for 1 hour. - Down to 1 g of the metabolite per ml of urine was easily detected. - Recovery of the metabolite at the extraction stage was ≃ 100% but only 30 to 40% was recovered by the prepared TLC.	99
3% of OV-1			- Tetracosane was used as internal standard. - The cocaine HCl contents of 6 samples analysed ranged from 6 to 100%. - Recoveries were 98.7 to 103%. - Coefficient of variation were 0.89 to 3.16. - The method was adopted as official first action.	122

Continued Table (10).

Column	Carrier gas	Detector	Remarks	Ref.
2-M glass column packed with 5% of polyoxyethylene glycol 20 M on Chromosorb W (100-120 mesh)	- Nitrogen 30 ml/min^{-1} - Oven temperature is programmed to 230° at 8° min^{-1}.		- Used for the identification narcotics and psychotropics through pyrolytic GC. - The sample in aqueous solution (1 to 2 µl containing 50 to 100 µg of salt of the drug), is placed on the filtered wire and dried in a stream of hot air (100°). Pyrolysis is carried out at 610° for 10 s, and the product is then separated in the column. - Salt analysis is preferred (because of reduced volatility).	123
4 ft X 0.125 in. packed with Chromosorb W (100-120 mesh) coated with 3% of OV-1.	Helium 30 ml/min^{-1} temperature 230°.	Nitrogen-phosphorous detector	- Blood + internal standard is buffered at pH 9 and extracted with 1-chlorobutane and the extract (plus HCl) is evaporated at 60° under nitrogen. - Calibration graph are rectilinear for 0.2 to 1 µg ml^{-1} for the drugs (cocaine and other basic drugs).	124
Glass column (20 m X 0.35 mm i.d.) wall-coated with SE-30.	Nitrogen 1.4 ml/min^{-1} temperature programming 5 min^{-1} for 50° or 70° to 250°.	Flame ionisation	- Ethyl morphine is used as the internal standard.	125

Continued Table (10)

Column	Carrier gas	Detector	Remarks	Ref.
A Pye 104 Chromatograph with a column (1.5 m X 4 mm) of Gas-Chrom Q (100-120 mesh) supporting 3% of OV-17	Nitrogen temperature 220°.		Rapid method for the determination of cocaine.	126
Glass column (6 ft X 2 mm) packed with a 1:1 mixture of 3% of OV-17 on Varaport 30 (80 to 100 mesh) and 5% of SE-30 on Chromosorb W (80 to 100 mesh)	Helium 30 ml/min^{-1} The column was programmed (2° min^{-1} for 190° to 240°.	Flame ionisation	- Used for the determination of the drug, heroin and morphine. - The powdered sample was extracted with chloroform : methanol (3:1) containing resmethrin as internal standard.	127

Continued Table (10)

Column	Carrier gas	Detector	Remarks	Ref.
A silanised - glass column (2.4 m X 2 mm) packed with 6% of OV-1 on Chromosorb W AW - DMCS (100 to 200 mesh).	Nitrogen 25 ml/min^{-1} 220°	Flame ionisation	- Used for the chromatographic analysis of the cocaine of Erythroxylum coca from three location in Peru. - Androst-4-ene-3,17-dione was used as internal standard.	128
Conventional OV-1 column	Helium (205°)	Nitrogen - phosphorous detector	- Cocaine and benzoyl ecogonine were quantitated after JETUBE extraction and derivatisation. - The propyl ester of benzoyl ecgnonine is used as the internal standard.	130
(74 cm X 2 mm) column packed 2% of OV-101 on Gas-Chrom-Q AW DMCS (100-120 mesh)	Helium (20 ml min^{-1}) (temp-programming from 140° to 240° at 10° min^{-1})		- Used to determine ecgnonine methyl ester, a major metabolite of cocaine, in urine, after oral administration of cocaine. - Phencyclidine is the internal standard. - The effluent is monitored by 70 eV m.s. at m/e 82 for cocaine and its ester and at m/e 200 for the internal standard (phencyclidine).	131

Other gas chromatographic procedures for the separation and identification have been published (53,56,81,99,129,132-150).

9.6.4 Gas Chromatography-Mass Spectrometry (GC-MS)

Cocaine has been determined by GC-MS by several authors:-

a) Jindal and Vestergaard (151) have reported a method for quantitation of cocaine and its principal metabolite, benzoylecgonine by glc-ms using stable isotope-labelled analogues as internal standards. Extract the cocaine from urine at pH 9 into chloroform and then into 0.1N HCl, adjust the solution to pH 9 with aqueous ammonia, re-extract into chloroform, evaporate the extract to dryness at 40° under nitrogen, and dissolve the residue in benzene. Analyse a portion of the solution of glc-ms on a glass column (1.8 m X 2 mm) containing 1.5% of OV-1 on Gas Chrom Q (100 to 200 mesh) and operated at 205° with helium as carrier gas (20 ml min^{-1}). Use N-(trideuteromethyl) cocaine as the internal standard, and compare the ion intensities at m/e 303 and 306. Extract benzoylecgonine and the internal standard, N-(trideuteromethyl)-benzoyecogonine, from urine at pH 7 into chloroform : isopropyl alcohol (4:1), evaporate the extract to dryness at 60° under nitrogen, convert benzoylecgonine into the ethyl ester with ethanolic diazomethane, evaporate at 40° under nitrogen, and dissolve the residue in chloroform. Extract the solution with 0.1N - HCl, adjust the aqueous phase to pH 9, and extract with benzene, evaporate the extract to dryness at 40° under nitrogen, dissolve the residue in benzene, and submit on aliquot to glc-ms as far cocaine, but compare intensities at m/e 317 and 320. About 2 ng ml^{-1} of cocaine and 5 ng ml^{-1} of benzoylecgonine can be determined with a precision of about 5%.

Lawry et al (57) have identified two novel cocaine metabolites in bile by gas chromatography and by gas chromatography-mass spectrometry in a case of acute intravenous cocaine overdose. The drug is extracted from the sample into 1-chlorobutane and back-extracted into weak acid. The acid extract is made alkaline and the compound is extract into chloroform for glc with 3% of OV-1 or OV-17 as stationary phase. For glc, ms, the OV-1 column is used, with temperature programming and electron-impact m.s. The compounds identified include cocaine, the known metabolites, methylecgonine, norcocaine, and benzoylecgonine, and also a hydroxycocaine and methylecgonidine.

Chinn et al (152) have described a gas chromatography-chemical ionisation mass spectrometry of cocaine and its metabolites in biological fluids. The sample, e.g. blood, urine, aqueous tissue homogenate or vitreous humour, is treated with sodium fluoride to inhibit enzymic hydrolysis of cocaine. The one portion (plus trideuterated cocaine as internal standard) is made alkaline with K_2HPO_4 and extracted with toluene-heptane-isoamyl alcohol (7:2:1); this extract contains cocaine and norcocaine. A second portion (plus trideuterated benzoylecgonine as internal standard) is saturated with sodium chloride and extracted (at pH 7) with chloroform-isopropyl alcohol (9:1); the extract is evaporated at 60°, and the residue is treated by a method similar to that of Jain et al (115) to form the propyl ester of benzoylecgonine which is purified by extraction. The final extract contains cocaine and the ester of benzoylecgonine. Each extract is analysed by glc at 205° [column 1.2 m X 2 mm; 3% of OV-1 on Gas Chrom Q (100 to 120 mesh); methane (≃20 ml min^{-1}) as carrier gas and reagent gas for the subsequent 120 eV m.s.]. The mass spectra are monitored at m/e 304 for

cocaine, 307 (for deuterated cocaine); 290 for norcocaine and 332 and 335 for the derivatives of benzoylecgonine and deuterated benzoylecgonine respectively.

Jindal et al (153) have also determined cocaine and its biologically active metabolite, norcocaine, in human urine. The sample (1 ml) was treated with [$^2H_3$]-norcocaine (56 ng) and, after adjustment to pH 8.5, was extracted with cyclohexane. The residue from evaporation of the extract was treated with trifluoroacetic anhydride, the mixture was evaporated and an aliquot of a solution of the residue in benzene was subjected to g.c.-m.s. with use of a glass column (1.8 m X 2 mm) packed with 1.5% of OV-1 on Gas Chrom Q (100 to 120 mesh) and operated at 205°. The mass spectrometer was operated in the selected-ion monitoring mode at 70 eV, and the intensities of the ions at m/e 303 and 306 and m/e 263 and 266, were used to measure cocaine and norcocaine, respectively.

Jindal et al (154) have also published a gas-liquid chromatographic - mass spectrometric determination of Lidococaine (Lignocaine) in an illicit sample of cocaine. The sample, in chloroform, was injected on to a glass column (1.8 m X 2 mm) silanised with 5% of dichlorodimethylsilane in toluene, packed with 3% of OV-17 on Gas Chrom Q (100 to 200 mesh) and operated at 200°, with flame ionisation detection. Two peaks were resolved, one was due to cocaine and the other to lignocaine as was shown by combined g.c.-70 eV m.s. (the molecular ion was at m/e 234 and the base peak was at m/e 86).

Clark (155) has described a mass-spectral quantitation method for the analysis of cocaine hydrochloride in powders. A solution containing 4 mg each of cocaine hydrochloride and [$^2H_5$]-cocaine

in 25 ml of methanol is analysed by g.l.c.-m.s. on Finnigan model 9500 and model 3300 instruments linked by a jet separator. A 0.2 µl portion of solution is injected (via a port at 240°) into a glass column (120 cm X 2 mm) packed with 3% of OV-1 on Gas Chrom Q (100 to 120 mesh) and operated at 190°, with helium as carrier gas (40 ml min^{-1}). The spectrometer is operated in repetition scan mode (every 2s) from m/e 75 to 310.

Lewin et al (156) reported a combined g.c.-m.s. of cocaine and its three isomers (four isomers) and of the corresponding ecgonine methyl esters, with use of a column (1.8 m X 2 mm) of 2% of OV-17 and chemical ionisation, with isobutane or NH_3 as reagent as, is studied; fragmentation patters are discussed in detail.

Other g.c.-m.s. methods have also been published (62, 81, 157-159).

9.6.4 High-Pressure Liquid Chromatography (HPLC)

Several HPLC systems for the identification and analysis of cocaine have been reported in the literature:-

- Olieman et al (160) reported a method for analysis of cocaine, pseudococaine, allococaine and allopseudococaine by ion-pair reverse-phase high-performance liquid chromatography. The compounds can be separated by liquid chromatography on a column of octadecylsilyl-silica with the addition to the eluent of n-heptanesulfonate and identified by peak area measurements at different ultraviolet wavelength.

- Masoud and Krupski (161) have devised an HPLC method for the analysis of cocaine in human plasma after being is used as an anaesthetic for nasal surgery. Blood is collected in a tube containing sodium fluoride and D-glucose citrate phosphate

solution (pH 5.7), and the plasma (with amethocaine HCl added as internal standard if desired) is made alkaline with sodium carbonate solution and extracted with ether. The drugs are back-extracted into acetic acid, then the acid layer is made alkaline and the drugs are extracted into hexane. This extract is evaporated under nitrogen at 40° and the residue is dissolved in the mobile phase [methanol : 0.05 phosphate buffer (pH 6.6) (3:1)] for h.p.l.c. at 40° on a column (25 cm X 2.6 mm) of Perkin-Elmer ODS-HC SIL-X-1 fitted with a brownless RP-18 MPLC guard column's elution is at 0.8 ml min^{-1} and detection is at 232 nm. Retention times are 5.2 min for cocaine and about 8 min. for amethocaine.

- Poochikian and Cradock (162) have determined cocaine in the presence of its hydrolysis product. The drugs were separated from each other and from the internal standard (4-chloropyridine) by h.p.l.c. on a column (30 cm X 4.6 mm i.d.) of μ Bondapak C18 (10 μm) operated at ambient temperature with 15 mm phosphate buffer (pH 3)-acetonitrile (3:1) as the mobile phase (0.8 ml min^{-1}) and detection at 235 nm. The detection limits were 3 ng for cocaine.

- Lewin et al (156) have identified and quantitated isomeric cocaines by HPLC. Cocaine is satisfactorily separated from its three isomer by h.p.l.c. on a column of partisil-10 PXS operated with isopropyl alcohol-heptane-diethylamine (25:75:0.1) as mobile phase at a flow rate increasing (during 12 minutes) from 0.48 to 4 ml min^{-1}; the elute is monitored at 230 nm. Calibration graphs are based on peak areas relative to those of N N-dibenzylbenzamide (the internal standard).

- Evans and Morarity (163) have reported the analysis of cocaine and its metabolites

HPLC. The plasma or tissue homogenate is mixed with aqueous internal standard (Lignocaine) and solid sodium fluoride (to inhibit enzymic hydrolysis of cocaine and norcocaine), the pH is adjusted to 9 (carbonate buffer solution) and the mixture is extracted with chloroform-isopropyl alcohol (3:2). The extract is evaporated under nitrogen at 40° and a solution of the residue in H_2O is submitted to h.p.l.c. on a column (30 cm X 4 mm) of μ Bondapak C18, with water - acetonitrile - methanol (8:1:1) containing 1% of acetic acid and 0.3M in EDTA as mobile phase (2 ml min^{-1}) and detection at 235 nm. Retention time for cocaine 9.7, benzoylecgonine 2.9, Lignocaine 4.2 and norcocaine 11.1 min.

- Fletcher and Hancock (164) have reported potential errors in benzoylecgonine and cocaine analysis. Cocaine HCl solution (50 μg ml^{-1}) were adjusted to pH values between 2 and 9.4 and were analysed immediately, or after being set aside for upto 6.75 hours by h.p.l.c. on a column (10 cm X 4.6 mm) of Hypersil 5-ODS (5 μm) with aqueous 55% methanol adjusted to pH 3.8 with H_3PO_4 as mobile phase (2 ml min^{-1}). Benzoylecgonine (retention time 1.4 minutes) and cocaine (retention time 3.4 minutes) were detected at 232 nm.

- Noggle et al (165) have published a liquid chromatographic procedure for identification of cis and trans- cinnamoylcocaine in illicit cocaine. A methanolic solution of the sample was analysed by h.p.l.c. with use of a stainless steel column (30 cm X 4 mm) of μ Bondapak-C18, with phosphate buffer (pH 3)-methanol (2:1) as mobile phase (2 ml min^{-1}) and with two ultraviolet detectors, at 254 and 280 nm, respectively, in series. The relevant fractions were collected, made alkaline with aqueous ammonia and extracted with dichloromethane. Residues obtained on evaporation were dissolved

separately in methanol for the analysis by ultraviolet spectrophotometry and by mass spectrometry.

Development of a standardized analysis strategy for basic drugs using ion-pair extraction and high-performance liquid chromatography, philosophy and selection of extraction technique have recently been reported (166).

Others HPLC methods have also been reported (167,168).

9.7 Radio-immunoassay

Mule' et al (169) reported the evaluation of the radio-immunoassay for benzoylecgonine (a cocaine metabolite) in human urine. The ^{125}I-radio-immunoassay (RIA) for benzoylecgonine in urine was evaluated by comparison with gas liquid chromatograph and thin-layer chromatography and the enzyme-multiplied immunoassay technique. By radio-immunoassay, a statistically significant concentration, 2 µg/litre, was observed for urinary benzoylecgonine. The coefficient of variation for the radio-immunoassay was 2.58 ± 0.38% interassay and 2.20 ± 0.14% interassay. There was cross-reactivity with cocaine (more reactive than benzoylecgonine and other members of the tropane family of alkaloids. There was agreement between results by radio-immunoassay and gas liquid chromatography in 95.5% of the samples, between radio-immunoassay and thin-layer chromatography in 87.0% and between radio-immunoassay and enzyme-multiplied immunoassay technique in 84.5%. The percentage of true false-positive was 3.5% for the radio-immunoassay in comparison to gas-liquid chromatography, 8.8% in comparison to thin-layer chromatography and 9.1% in comparison to enzyme-multiplied immunoassay technique. True false-negative were insignificant (0. to 1.0%). Gas liquid chromatography and radio-immunoassay results correlated highly (ϕ = 0.908). Gas-liquid chromatography, therefore, was the best comparison method for the evaluation study. Radio-immunoassay for benzoylecgonine is sensitive, reproducible and reliable for the detection of cocaine in urine.

Budd (170) reported a cocaine radio-immunoassay-structure versus reactivity. He tested several alkaloids for benzoylecgonine antibody-binding activity in the Roche r.i.a kits. Benzoylecgonine has the optimum antibody-binding activity; change of any of the substituents (except esterification of the carboxy-group) reduced the binding, coca-ethylene was the only drug that interferred with the Roche assay at therapeutic or overdose levels, but it was seldom encountered under these conditions. Thus the kit was considered to be suitable for assaying cocaine and its metabolites.

Baum-gartner et al (171) published a method for radio-immunoassay of cocaine in hair. The drug was detected in hair of 13 patients from a drug-abuse clinic who acknowledged having used cocaine in varying amounts during the last six months. A correlation was observed between the amount of cocaine used and the quantity trapped in the interior of hair grown during the six month period. In contrast to hair analysis, urinalysis by thin-layer chromatography was negative in all cases. Indicating that, cocaine has not been used by the patients within 48-72 hours before the urine collection. Hair analysis thus appears to be far superior to urinalysis for establishing histories of drug use.

Other immunoassay methods have also been reported (110, 172-174).

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EPHEDRINE HYDROCHLORIDE

SYED LAIK ALI

Ephedrine Hydrochloride

Ephedrine and ephedrine hydrochloride have been mostly used synonymously in this monography. Ephedrine is quite often cited in literature but it is presumed to be valid also for ephedrine hydrochloride. Aqueous or acidic solutions of ephedrine hydrochloride are often converted into ephedrine before other chemical or chromatographic operations and measurements. Casually ephedrine sulfate has been also included in this monograph. Physical properties, spectra etc. are given for ephedrine hydrochloride.

1. History

Ephedra is a Phanerogame-Gymnosperme from the family of Gnetaceen. There are 30 different types of this plant-species known which grow in Asia, mediteranian countries and America. Specially ephedra vulgaris, ephedra equisetina and ephedra sinica contain ephedrin with its other isomers. A certain ephedra species has been used in ancient chinese medicine since ages. Already in 5000 B.C. an ephedra-plant was widely used in China under the name Ma-Huang. Ma-Huang has been mentioned as medicine of moderate therapeutic range in the first chinese pharmacopoe, published under the government of Shen Lung in 1760 B.C. A detailed description of the plant and its pharmacological action is given in the chinese pharmacopoe of modern times in 1596. According to this pharmacopoe Ma-Huang was applied as Diaphoreticum, Antipyreticum and Sedativum for the treatment of coughs and colds as well as a stimulant for blood-circulation (1). These therapeutic effects were mostly confirmed later for ephedrin which was discovered and isolated in pure form by Nagai (2) in 1887 as a main alkaloid of ephedra from ephedra sinica.

2. Nomenclature

Ephedrine hydrochloride is (IR,2S)-(-)-β-hydroxy-N α-dimethyl-phenethylammonium chloride. The formula is illustrated at the next page.

3. Description

3.1. Name, Formula, Molecular weight

Ephedrine Hydrochloride
$C_{10}H_{15}NO$ HCl 201.70

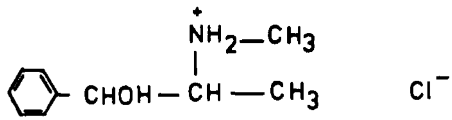
3.2. Appearance, Colour, Odour

Colourless crystals or white, crystalline, orthorohombic needless or powder, odourless, with a bitter taste.

4. Synthesis

Ephedrin was synthesised for the first time by Späth (3) as illustrated in Fig. 1. Racemic ephedrin was then further split up into its components. Addition of methylamin to 1-phenylpropylenoxyd leads also to the synthesis of ephedrin (4). Ephedrine could further be synthesised through amination of α-Brompropiophenon with methylamine or benzylmethylamine. α-Brompropiophenon was synthesised through bromination of propiophenon and propiophenon itself was prepared with Friedel-Crafts reaction using benzene and propoyl chloride (5). The resulting product methylaminoketon or benzylmethylaminoketone was then hydrogenated in presence of palladium as catalyst and a racemic mixture of ephedrin was obtained (Fig. 2). This was further reacted with sodium dibenzoyl tar-

Ephedrine Hydrochloride



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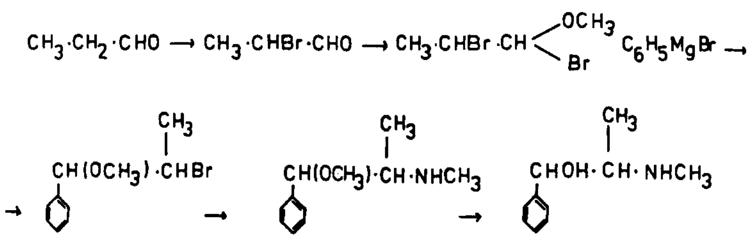


FIG. 1

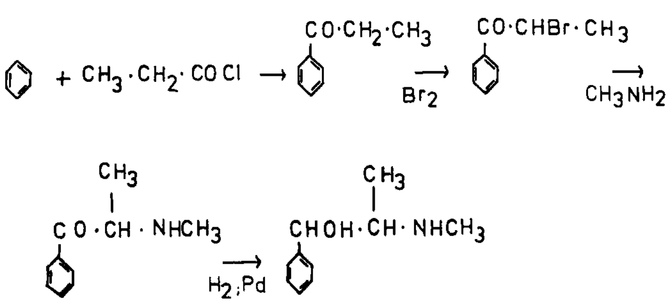


FIG. 2

trate to convert ephedrine into its optical isomers. Dibenzoyl tartrate of (-)ephedrine was then treated with hydrochloric acid to give (-)ephedrine hydrochloride in 60 % yield. This process was used on an industrial scale by C-H-Boehringer Company in Germany during second world war (5). Later on this method was further modified by using dibenzoyl(+)-tartaric acid or mandelic acid for the resolution of racemic α-methylamino-propiophenone into its optical isomers in 90 % yield. The resulting product was then catalytically hydrogenated to (-)ephedrine (6). A biochemical process for the production of optical active (-)ephedrine-isomer is applied by Knoll Company in Germany. L-phenyl acetyl carbinol is obtained through an acyl condensations with benzaldehyde during the fermentation process of a Melasse-solution. This compound is then reacted with methylamine and simultaneously reduced to (-)ephedrine in presence of active aluminium or a platinum catalyst (Fig. 3). Ephedrine is then converted into ephedrine hydrochloride which is recrystallised from water into optically pure isomer (7,8). Upon heating ephedrine hydrochloride is decomposed into phenyl ethyl ketone and methyl amine.

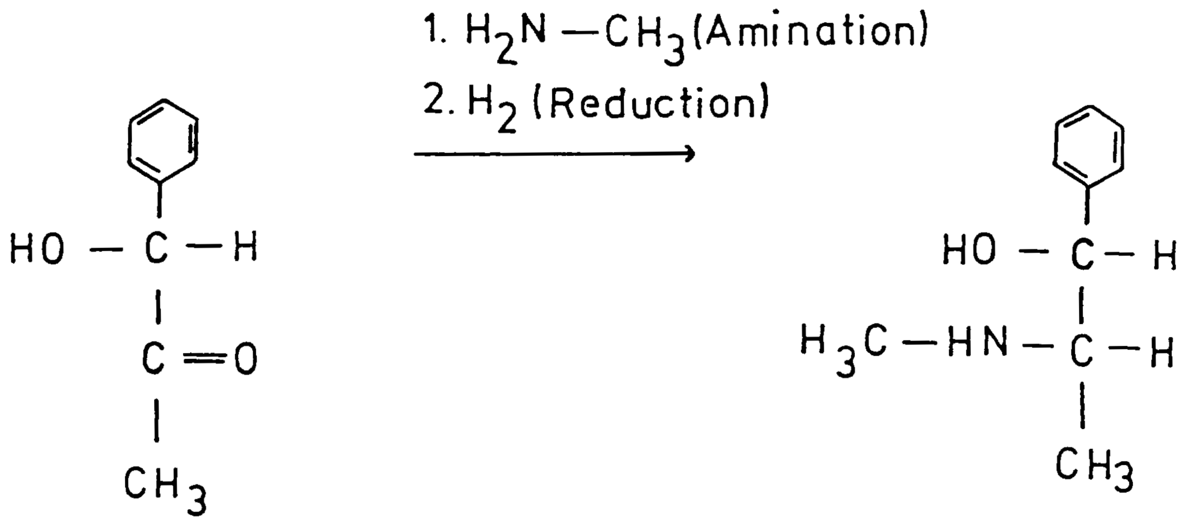
5. Physical properties

5.1. Solubility

It is freely soluble in 4 parts of water, and in 17 parts of alcohol (96 %), very slightly soluble in chloroform and practically insoluble in ether (9).

5.2. Loss on Drying

Not more than 0.5 %, determined with 1.00 g by drying to constant weight in an oven at 100° - 105°C (9).



1. H2N —CH3 (Amination)
2. H2 (Reduction)
HO — C — H
C = O
CH3
HO — C — H
H3C — HN — C — H
CH3

FIG. 3

5.3. Melting point

217° - 220°C (9)

5.4. Specific optical Rotation

-33.5° to -35.5°, determined by dissolving 5.00 g in sufficient water to produce 50.0 ml, diluting 10 ml to 20 ml with water and calculated with reference to the dried substance (9).

5.5. Sulphates

100 ppm with limit test for sulphates (9).

5.6. Sulphated Ash

Not more than 0.1 %, determined with 1.0 g (9).

5.7. pH-Value

The pH of a 0.5 % aqueous solution is 5.9.

5.8. Dissociation constant

The Pk_a of ephedrine hydrochloride in water at 20°C is reported to be 9.68 (11). This value is attributed to the ammonium cation of the molecule.

5.9. Ultraviolet Spectrum

The ultraviolet spectra of ephedrine hydrochloride were taken with a Perkin-Elmer UV spetrophotometer 571 at a concentration of

0.5 mg/ml in methanol, 0.1N HCl and 0.1N NaOH. In all three solvents the spectra show absorption bands at almost identical wave lenghts of 250, 256 and 262 nm.

The molecular extinction coefficients and $A^{1\%}_{1cm}$ of ephedrine hydrochloride are reported to be (12)

	Methanol	0.1N HCl	0.1N NaOH
Absorption Maximum	250 nm	250 nm	251 nm
	256 nm	256 nm	257 nm
	262 nm	262 nm	263 nm
$A^{1\%}_{1cm}$	8.4	7.4	7.8
	10.8	9.4	9.7
	8.2	7.2	7.4
ϵ	170	150	160
	220	190	195
	165	145	150

The UV spectra are shown in Fig. 4.

5.10. Infrared Spectrum

The infrared spectrum of ephedrine hydrochloride is given in Fig. 5. The spectrum was obtained with a Perkin-Elmer 1420 Ratio Recording Infrared Spectrophotometer from a KBr pellet. The structural assignments may be correlated with the following band frequencies:

Frequency (cm^{-1})	Assignments
3330	Stretching vibrations of OH
2700-2840	Amine halide salt stretching bands
2480	NH_2^+ stretching
1450 and 1490	Characteristic vibrations of the aromatic ring
750 and 698	C-H out of plane deformation, monosubstitued benzene

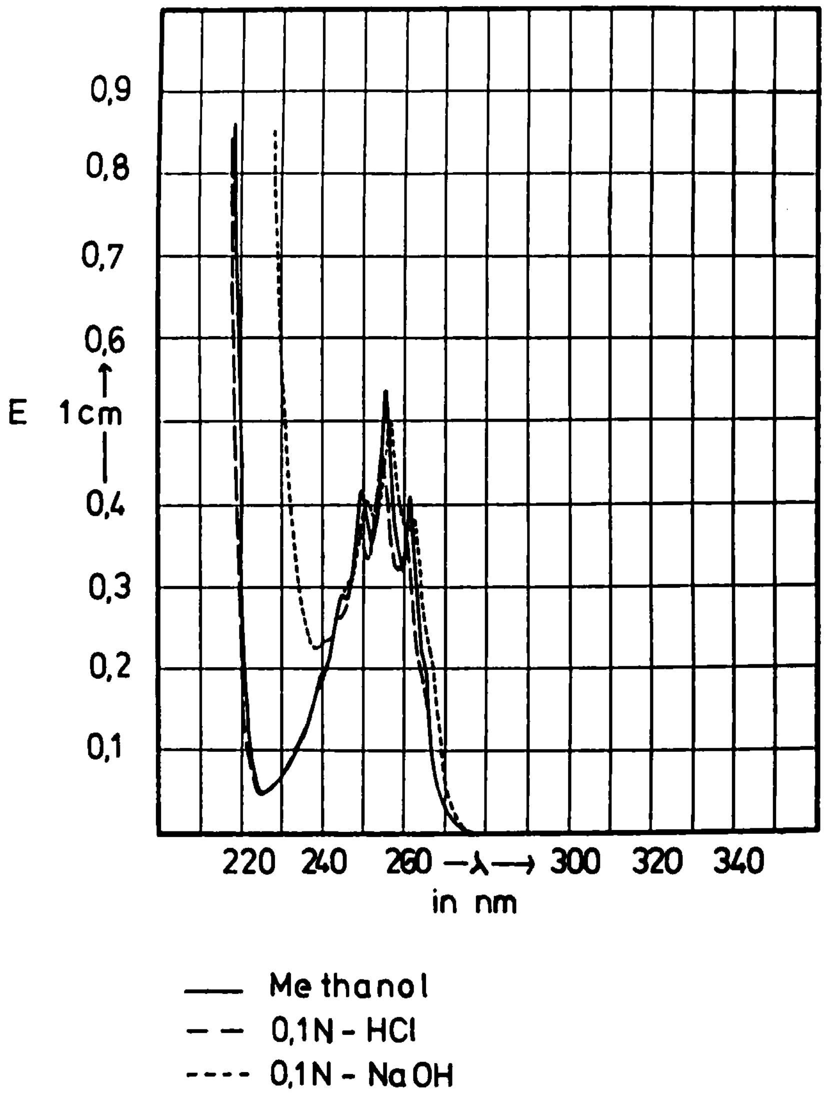


FIG. 4

'UV SPECTRUM OF EPHEDRINE HYDROCHLORIDE IN DIFFERENT SOLVENTS

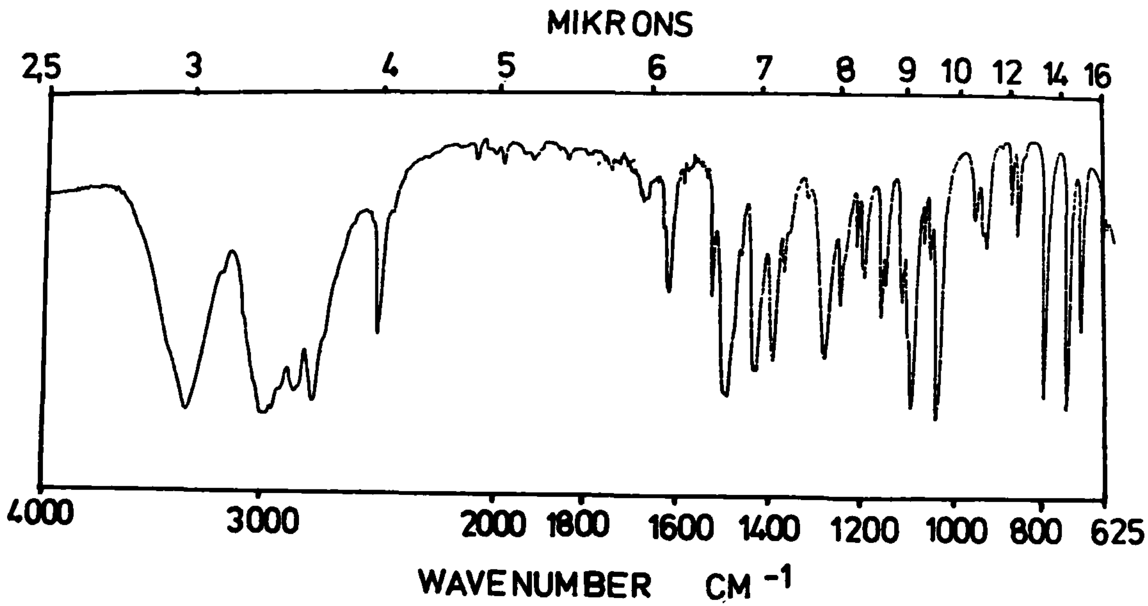


FIG. 5
IR SPECTRUM OF EPHEDRINE HYDROCHLORIDE, KBR PELLET, PERKIN-ELMER 1420 SPECTROPHOTOMETER

5.11. Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum of ephedrine hydrochloride was obtained with a Varian T-60 NMR spectrometer in D_2O. The following spectral assignments are made for the spectrum reproduced in Fig. 6.

Chemical shift	Assignment
1.20 doublet	CH_3 at CH
2.90 singlet	CH_3 at NH_2
3.60 multiplet	CH at NH_2
5.28 doublet	CH at OH
7.53 singlet	aromatic protons

OH and NH_2 deuterated.

5.12. Mass spectrum

In the highest mass regions molecular ion peak ist not observed. Prominent ion-peaks observed are m/e, 31, 59, 78 and 108. The mass spectrum of ephedrine hydrochloride is given in Fig. 7.

Instrument:	Varian Mat 44
Sample temperature:	(direct inlet) 50°C - 250°C in 5 min
Source temperature:	250°C
Electron energy:	80eV

Some ions of this spectrum can be correlated to the structure as following:

$M-C_6H_5CHOHHCl = 59$; $M-CHOHCHCH_3HCl = 78$ (with NH CH_3 above the CH of CHOHCHCH₃HCl)

$M-CHCH_3HCl = 108$
$\quad NHCH_3$

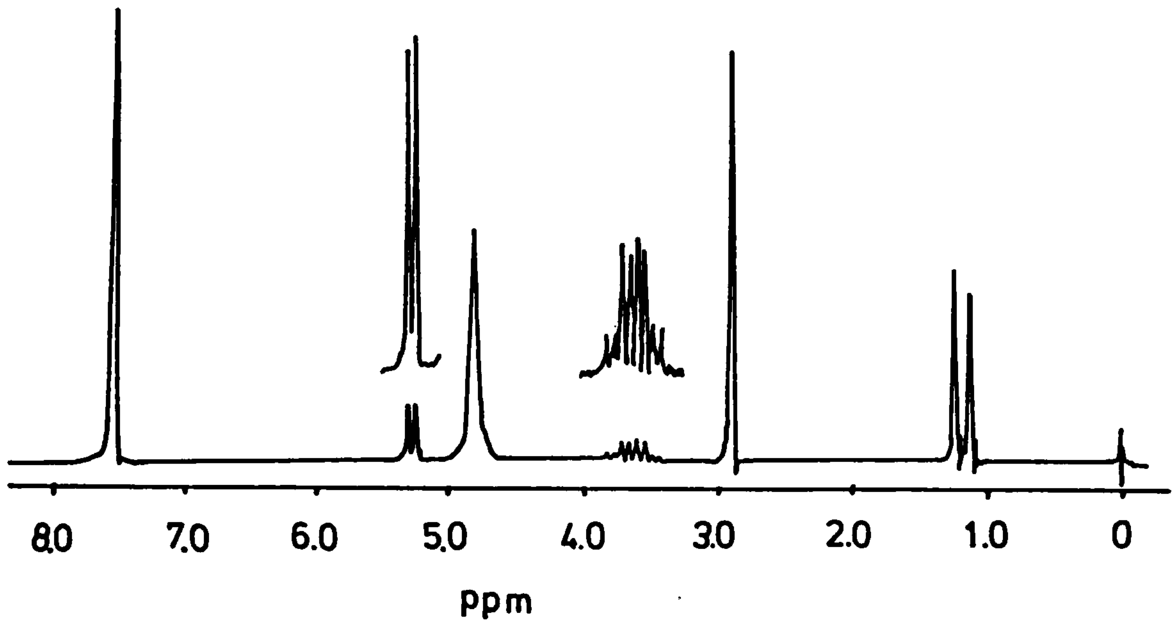


FIG. 6

NMR SPECTRUM OF EPHEDRINE HYDROCHLORIDE, VARIAN T 60 SPECTROMETER

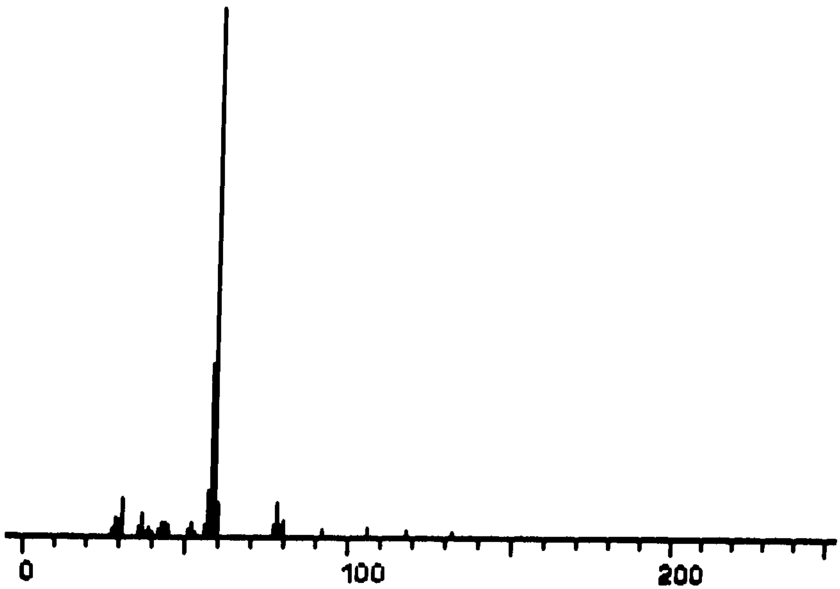


FIG. 7

MASS SPECTRUM OF EPHEDRINE HYDROCHLORIDE

6. Stereochemistry

Ephedrine has two asymmetric centres; there exist four steroisomers and two inactive forms $\pm$ ephedrin and $\pm$ pseudoephedrin. The erythro-configuration of asymmetric centres in (-)ephedrine was confirmed by Freudenberg (13) through the synthesis from D(-)mandelic acid and L(+)-alanin. According to the Cahn-Ingold-Prelog nomenclature the absolute configuration of asymmetric carbon atoms in (-)ephedrine could be defined as 1R,2S. Hyne has found with nmr measurements the dieder angle of 90° between OH and $NHCH_3$ in ephedrine base (14). Gauche-Confirmation A and B are strongly favoured in free ephedrine base and its salts (15). Ephedrine hydrochloride dissolved in D_2O lies 90% in A and B forms and 10% in trans-form C (Fig. 8). Testa (16) found good agreement of CD and ORD results with nmr values.

7. Colour and Identification Reactions

To 10 mg ephedrine hydrochloride in 0.1 ml water when 0.2 ml of copper sulphate solution (12.5%) and 1 ml of strong sodium hydroxide solution (10 N) are added, a violet colour is produced. When this solution is shaken with 2 ml ether, the ether layer turns purple and the aqueous layer remains blue (17). 50 mg of the substance dissolved in 1 ml water is mixed with 4 ml 0.1N NaOH, shaken with 3 ml CCl_4 for ten seconds and then allowed to stand for 2 minutes. The organic layer is separated and treated with few copper turnings. A turbidity appears rapidly which turns in few minutes into a copious precipiate (18, 19). L- and D,L-ephedrines could be distinguished through their crystalline modification. An aqueous ephedrine salt solution is acidified with 3 drops of 15%

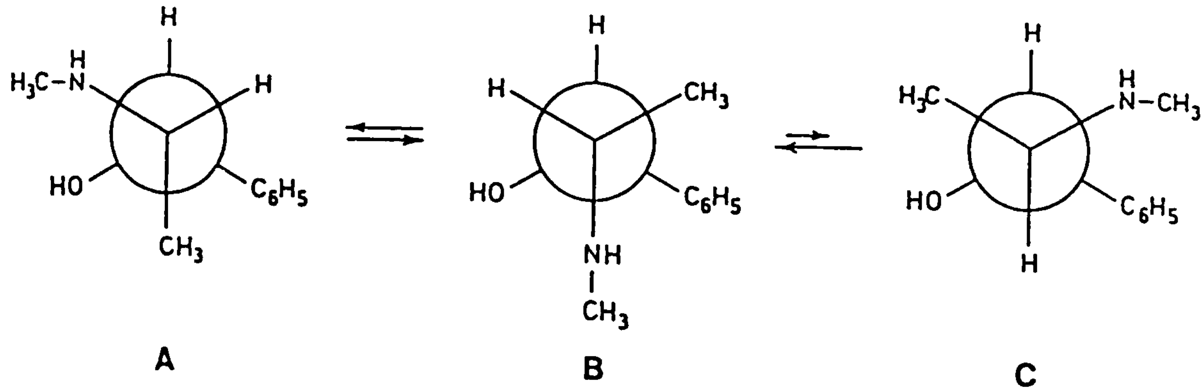


FIG. 8

STEREOCHEMISTRY OF EPHEDRINE HYDROCHLORIDE

H_2SO_4, 2 ml potassium iodobismutate solution (prepared by mixing 2 parts of 7 % basic bismuth nitrate in nitric acid, 1 part potassium iodide and making upto volume of 10 ml with water) are added and left to stand overnight. L-ephedrine is identified under microscope with needle-like crystals, whereas D,L-ephedrine is visible as dark-red prisims (20). Ephedrine hydrochloride gives in weak alkaline solutions with ninhydrine a violett colour (21).

8. Stability and Degradation

Decomposition of (-)-ephedrine was less than 1% after the prolonged passage of air through cold (20°C) or refluxing neutral or basic aqueous solutions (0.2% W/V ephedrine hydrochloride in phosphate buffer pH 7.4 or in 1% sodium hydroxide). On exposure to heat ephedrine hydrochloride is decomposed in phenyl ethyl ketone and methyl amine. GLC-MS provided almost the sole means of identifying some of the breakdown products of ephedrine due to their similar GLC properties and lability on tlc. Significant losses occurred during the extraction of small quantities of ephedrine from aqueous media using either regular or analytical grades of diethyl ether. The losses were, at least in part, caused by reaction of the ehedrine with aldehydic impurities in the ether. The addition of n-butanol to the ethereal extract before evaporation reduced the "breakdown" that occurred if the extracts were allowed to boil dry in the water bath. For this reason the routine use of aldehyde-free n-butanol is of value.

Different substituted oxazolidines were identified through GLC-MS as break-down products. Consistent low levels of ephedrine "breakdown" were achieved by prior washing of the

ether with 10% sodium metabisulphate solution followed by 1N hydrochloric acid and finally with sodium hydroxide,5N. Negligible decomposition was observed on refluxing ephedrine (0.5%) in ether sturated with aqueous 20% NaOH for 8h or ephedrine (8%) in ethanolic sodium hydroxide for 3h. Ephedrine base stored in ether (100 mg/ml) at room temperature in light for several weeks decomposed to give oxazolidines. This is in contrast to the small amount of decomposition that occurred upon ultraviolet irradiation of aqueous solution of ephedrine. Solutions of ephedrine base (3% W/V) in ether or benzene were extensively degraded by ultraviolet light over 18h.

The major decomposition products yielding peaks upon glc examination after extraction of ephedrine solutions with ether and concentration of these extracts arise from addition and condensation of ephedrine with acetaldehyde, propionaldehyde and formaldehyde impurities in the solvent. Oxidation of (-)-ephedrine base with nickel peroxide, active silver carbonate and with active manganese dioxide gave benzaldehyde, a mixture of oxazolidines and 2-methyl-amino-1-phenyl-1-propanone as oxidation products. The degradation products were identified through glc and glc-ms analysis (22).

9. Methods of Analysis

9.1. Titrimetry

The assays of halogen salts of organic bases can be carried out either by a two-phase-titration or through non-aqueous titration of the substance. The two-phase titration applies ethanol 96% and chloroform as solvent, phenolphthalein as an indicator and 0.1N sodium hydroxide as the titrant. The deter-

mination of ephedrine hydrochloride in this medium is rather uncertain and gives about 3 % lower results (23). Ephedrine hydrochloride is determined in german pharmacopoeia (DAB 7) through dissolving it in water, acidifying with 3N nitric acid, additon of excess of 0.1N silver nitrate solution and back-titration of silver nitrate with 0.1N ammonium thiocyanate using ferric ammonium sulphate as an indicator (24). In non-aqueous medium either ephedrine hydrochloride is dissolved in glacial acetic acid, mercuric acetate and then few drops of crystal violet indicator solution are added. The solution is then subsequently titrated with 0.1N perchloric acid to an emerald-green end-point (USP XXI). Another method of non-aqueous titration is to dissolve the substance in warm mercuric acetate solution, to add acetone and then to titrate it against 0.1N perchloric acid using saturated solution of methyl orange in acetone as an indicator until a red colour is obtained (26, 27). An indirect non-aqueous titrimetric method was devised for the determination of hydrochlorides of nitrogen-bases. The chloride-ion interference was prevented without the use of mercuric acetate reagent. The method depends on the treatment of a solution of the hydrochloride of the organic base with an excess of standard perchloric acid solution in acetic acid and the hydrogen chloride displaced is removed by boiling. The excess of perchloric acid is determined by titration against the basic titrant sodium acetate in glacial acetic acid using either potentiometric or visual end-point detection with a crystal violet indicator. Potentiometric titrations showed that the point of the maximum inflection in the titration curve coincided with the appearance of the violet colour of the indicator. The mean percent recovery of 99.66 % obtained indicates that the proposed method is equivalent in accuracy and precision to the non-aqueous titrimetric

method most commonly used by official compendia (28). Sanchez determined ephedrine by its reaction with alkaline iodine solution at 50°C to form iodoform and titration of the excess of iodine in acid solution. Application of this method to standard solutions of ephedrine hydrochloride has given recoveries within ± 3% of the theoretical values. However, this method could not be applied to the determination of ephedrine HCl in cough mixtures due to interferences from other constituents (29). Horak and Gasperik used a method for the determination of ephedrine based on the liberation of methylamine by alkaline hydrolysis. Good results were obtained for the determination of ephedrine HCl in pharmaceutical injections and tablets (30).

9.2. Visible and UV-Spectrophotometry

UV spectrophotometric determination of benzaldehyde or substituted benzaldehydes formed by periodate oxidation of ephedrine and other drugs with vicinal hydroxyl and amine functions has provided simple and sensitive assay methods for them. Ephedrine hydrochloride in dilute HCl solution exhibits a molar absoptivity of 190 at its about 258 nm maximum. Oxidation of it to benzaldehyde affords an ϵ-value of about 14400 at its maximum at about 241 nm in hydrocarbon solvents, about a 75-fold again in sensitivity (31). Periodate oxidations are among the most elegant reactions used in organic chemistry, because they are often quantitative within minutes at room temperature in aqueous media. Malaprade introduced periodic acid as a reagent for the oxidation of 1,2-glycols in 1928 (32). Nicolet and Shinn first reported the use of periodate for oxidation of ethanolamine derivatives. They found that ethanolamines with primary or secondary amine functions were rapidly and quantitatively cleaved to al-

dehydes and ammonia or a primary amine (33). Wickstrom studied the rate of periodate oxidation of ephedrine as a function of pH, titrating excess of oxidant iodometrically. He found that periodate consumption was too slow to measure at pH 3.0, very slow at pH 6.0 and stoichiometric within 10 min at pH 7.5 or higher (34). Oxidation potential of periodate-iodate couple is -1.6V in acid solution and about 0.7 V in alkaline solution (35). Wickstrom found that the reaction products of periodate oxidation of ephedrine are benzaldehyde, acetaldehyde and methylamine. The determination of ephedrine via colorimetry of acetaldehyde distilled from the reaction mixture was suggested (34). Chafetz studied among others the periodate oxidation of ephedrine (36). It was quantitatively oxidised to benzaldehyde in 10 min in a bicarbonate medium. Extraction of benzaldehyde in n-hexane and its spectrophotometric determination was recommended. Spectrophotometry of the benzaldehyde produced by periodate oxidation will not distinguish between compounds such as ephedrine and phenylpropanolamine. It will further not discriminate steroisomers such as ephedrine (erythro-configuration) and pseudoephedrine (threo-configuration) (31).

Wallace conducted a reaction for the determination of ephedrine in biological samples at the temperature of boiling hexane, about 69°C, with a reaction time of 30 min. The hexane layer was separated, washed with dilute acid and benzaldehyde determined spectrophotometrically either as such or after conversion to its semicarbazone (37, 38). Chafetz (31) further showed that the carbonyl compounds analogs of ephedrine are oxidised to benzoic acid by periodate and do not interfere in the assay of ephedrine. It is further demonstrated that N-acetylephedrine does not react with periodate under the assay conditions.

An application of orthogonal functions to the UV spectrophotometric determination of ephedrine hydrochloride in tablets is reported (39). The method is applicable for the determination of a single substance in the presence of irrelevant absorption from excipients such as lactose, starch, sucrose, gelatin, talc, stearic acid and magnesium stearate. The choice of polynomial, number of points, wavelength range and intervals are illustrated. Glenn's method of orthogonal functions proved to be powerful in discounting irrelevant absorption contribution (40). A colorimetric method for the quantitative determination of ephedrine hydrochloride in presence of chlorpheniramine maleate and guaiacolsulfonate potassium in a cough syrup containing colouring agents is described (41). Ephedrine hydrochloride is assayed using bromothymol blue as a dye in which interference from chlorpheniramine maleate is taken into consideration. The extinction of the chloroform extract is measured at 420 nm against the reagent blank. A collaborative study for the on-column periodate reaction method for analysis of ephedrine in solid dosage forms is reported. Ephedrine is separated from water-soluble impurities and strong acids by elution from a weakly basic celite column, and further cleaned up by retention on a weakly acidic column while the weak acids, weak bases and organic-soluble neutrals are eluted. Ephedrine is eluted from the column after neutralisation with NH_3 and is converted to benzaldehyde via on-column periodate reaction and determined spectrophotometrically (42, 43). An UV spectrophotometric determination of ephedrine hydrochloride in an antiasthma capsule preparation containing aminophylline and amobarbital is reported (44). On a prepared column containing alginic acid ephedrine HCl is retained from an ethanolic solution; aminophylline and amobarbital pass through the column. Subse-

quently ephedrine HCl is eluted with 0.1N HCl and determined spectrophotometrically at 257 nm. A rapid second and fourth derivative UV spectrophotometric assay procedure is described for the determination of ephedrine or pseudoephedrine in pharmaceutical formulations. The method has been applied successfully to Ephedrine Elixir BP, Ephedrine HCl tablets BP, Ephedrine Nasal Drops, Paedriatric Belladonna and Ephedrine mixture, tablets, capsules containing aminophylline and amobarbital and coloured syrups containing tripoldine hydrochloride and codeine phosphate. A simple extraction procedure avoids interference from colouring agents in certain formulations. Specificity, accuracy and precision of the method has been assessed. The second derivative absorption spectrum of ephedrine HCl shows enhanced resolution of the fine structure. Discrimination against broad spectral bands in favour of narrow bands is one of the advantages in derivative spectroscopy. The second derivative assay of Ephedrine Elixir BP eliminates the interference of the broad absorption bands of excipients (45). Colouring agents or other coformulated drugs are removed from an ephedrine formulation by a simple solvent extraction procedure before the derivative spectroscopy. The recovery of ephedrine by the extraction method was 99.2 % (45). Ephedrine hydrochloride has been determined directly or after separation on cellulose thin layer plates photometrically using p-dianisidine in aqueous solutions at pH 7. The yellow colour has been measured at 424 nm. The method permits the determination of ephedrine in the range of 50-200 μg with a relative standard deviation of ± 4 % (46). Spectrophotometric determination of ephedrine is done at 629 nm after reacting it with periodic acid 3-methylbenzthiazolin-2-on-hydrazon (47). Organic bases like ephedrine are reacted with

bromthymol blue, subsequently extracted with chloroform or methylene chloride and the extinction determined at 420 nm. The dyestuff itself does not dissolve in organic phase. An alkaline reagent like tetraethyl ammonium hydroxide solution gives a blue coloured organic phase which could be determined at 620 nm ((48, 49). The coloured reaction product of bromcresol green with ephedrine can either be extracted at pH 5-6.2 with chloroform and the extinction is measured at 420 nm or the organic phase is further treated with an alkaline reagent and the extinction of the coloured aqueous solution is determined at 620 nm (50). Secondary alkyl amines react with carbon disulphide to give dithiocarbamates which form with cupric salts coloured chelates. Ephedrine hydrochloride has been determined spectrophotometrically at 437 after extracting its coloured chelate with chloroform (51, 52, 53).

9.3. Fluorimetry

A fluorimetric method has been developed for the determination of ephedrine hydrochloride as its dansyl derivative in ephedrine tablets. After dansylation the dansyl derivative is separated on a thin layer plate and determined directly densitometrically at 526 nm (54). In another method the dansyl derivative of ephedrine hydrochloride is eluted from the plate with ethanol and determined at its fluorescence maximum of 505 nm (55). Dansyl derivative of ephedrine has its excitation maximum at 354 nm and emission maximum at 476 nm (56).

9.4. Chromatographic Methods

Thin layer chromatography

Beckett and Choulis have separated ephedrine from other compounds such as adrenalin, noradrenalin etc., on cellulose, silicagel G and aluminium oxide plates using n-butanol + glacial acetic acid and water (40 + 10 + 50) or with water saturated with n-butanol as mobile phases. On cellulose tlc plates ephedrine and its salts give two spots. The use of alkaline mobile phases leads to only one spot (57). Choulis has used the mobile phases n-butanol-acetic acid-water (4+1+5) or phenol-0.1N HCl (85+15) with cellulose tlc plates for separation of ephedrine (58). Waldi recommended the converting of ephedrine to its corresponding triacetylderivative before applying it on silicagel G plates. Having decreased its hydrophilic character it was possible to develop the chromatogram with chloroform - methanol (9+1) as mobile phase. The Rf-value of ephedrine was found to be 0.51 (59). Ephedrine could be detected by spraying tlc plates with a 0.2 % ninhydrin solution in ethanol or n-butanol and heating the chromatogram to 130-140°C (60). The best modification with ninhydrin reagent is to use 0.3 % solution in n-butanol + 3 ml acetic acid (61, 62). A similar reaction can be carried out with Folin's reagent which re- sults in the formation of light rose-coloured spot for ephedrine (61).

A spectraldensitometric method is described for the determination of ephedrine in ephedra herb and ephedrine extracts. After tlc separation on silicagel G plates with the mobile phase n-butanol-glacial acetic acid-water (4+1+5) the chromoplate was dried, subsequently immersed in a 0.2 % ninhydrine solution in ethanol and finally dried at 110°C.

The violet spots of ephedrine at Rf-value 0.3 were evaluated densitometrically at a measuring wavelength of 540 nm and a reference wavelength of 680 nm (63).

Ephedrine hydrochloride in tablets along with other components has also been determined densitometrically after its conversion to the dansyl derivative and chromatographic separation on at 105°C activated silicagel 60 (Merck) tlc plates using benzene + ethanol + glacial acetic acid (90 + 10 + 1) as a mobile phase. Spectrodensometric measurement was carried on with a 526 nm filter. This procedure was on account of its great sensitivity and good reproducibility very useful in content uniformity determinations of ephedrine HCl in tablets and capsules (54). A tlc separation of ephedrine after its conversion to the corresponding dansyl derivative was carried on silica gel tlc plate with toluene + methanol + acetone (9+1+1) as mobile phase. The Rf-value for ephedrine was found to be 0.50. The spots were evaluated using spectrofluorimeter equipped with a tlc scanning attachment; excitation wavelength was 360 nm and the emission wavelength was set between 500-510 nm using a UV filter. The improved selectivity and sensitivity have permitted an analysis in 10-100-fold excesses of other drugs. Detection limits are reported in the range of 1-10 ng or better. The reproducibility of the method is limited by the derivatization step, but a relative standard deviation of less than 2 % could be obtained (56). Lang has applied cellulose tlc plates and mobile phases of n-butanol-glacial acetic acid-water (4+1+5) or n-propanol+benzene+glacial acetic acid + water (40+30+10+1) for the separation of ephedrine HCl in pharmaceuticals. The ephedrine spot was localised, scratched from the tlc plate, extracted with the solution of diazotized p-dianisidine

reagent, the solution was filtered and then measured in a spectrophotometer at 424 nm against corresponding blank reagent (46). Waldi has separated ephedrine from alkaloids of various other groups on a silica gel tlc plate with the mobile phase chloroform-diethyl-amine, (19+1), R_f:0.29 (64). Simultaneous detection of ephedrine along with a wide variety of commonly abused drugs in a urine screening program using tlc techniques has been reported. A detailed extraction procedure for the urine samples is given. Gelman precoated silica gel glass microfilter sheets with a layer thickness of 250 μm were used. Different solvent systems and detection reagents were used. The specific colour reactions obtained there may not be achieved on glass plates coated with silica gel. Solvent systems ethyl acetate-cyclohexane-methanol-ammonia (70+15+10+5) and ethyl acetate-cyclohexane-ammonia (50+40+0.1) were found suitable for the separation of ephedrine. Microgram amounts of ephedrine could be detected with the detection reagents 0.5 % ninhydrin in n-butanol, mercury(II)sulfate, iodoplatinate and iodine-potassium iodide solutions (65). NBD-Cl derivative of ephedrine was separated along with the corresponding derivatives of other drugs on silica gel G plate with solvent systems diethyl ether-benzene (1+1), ethyl acetate-cyclohexane (2+3) and ethyl acetate-cyclohexane (3+2) with R_f-values 0.42, 0.18 and 0.33 respectively. The spots showed yellow fluorescence in UV light 254 nm (66).

Separation and determination of ephedrine

along with other doping agents has been obtained through overpressurised TLC and HPTLC silica gel 60 F_{254} plates using an eluent n-butanol-chloroform-methyl ethyl ketone-water-acetic acid (25+17+8+4+6) with an external membrane pressure of 1.0 mPa. In comparison with the classical TLC, the resolution was improved, the development time was shorter and detection limit was lower. The quantitative evaluation was carried out with a scanner at 210 nm (67).

Ephedrine hydrochloride has been determined densitometrically at 270 nm after its separation on silica gel 60 HPTLC plates with the mobile phase ethyl acetate-glacial acetic acid-water (27+6+4) and nitration with nitrous gases (100 % nitric acid). After chromatography the HPTLC plate is heated in a drying chamber for 15 min at 160°C and then exposed while still hot to nitrous gases for 10 min. The relative standard deviation for ephedrine hydrochloride was found to be 1.75 % (68).

Paper Chromatography

By using papers impregnated with alkaline buffers, such as boric acid-NaOH, pH 10, and ether saturated with water as mobile phase it is possible to separate (+)ephedrine (R_f: 0.70) from (+)pseudoephedrine (R_f: 0.38) (60). Ephedrine and pseudoephedrine can be estimated after elution in the form of copper dithiocarbaminate complexes (60). Dittrich quantitates ephedrine in paper chromatography by using the iodine fixation properties of the ephedrine followed by iodometric determination after elution with KI solution (69). Other solvent systems for the paper chromatographic separation of ephedrine are n-butanol-acetic acid-water (4+1+5), R_f: 0.73 (61)

isobutanol+formic acid+water (100+12+10), R_f: 0.50 (70), n-butanol saturated with water, R_f: 0.64 (71), cyclohexane-diethylamine (9+1), R_f: 0.47 (72).

Gas Liquid Chromatography

The separation of optical amines by GLC can be achieved by using either an optically active stationary phase after making derivatives with a suitable optically inactive reagent or an optically active reagent such as N-trifluoroacetyl-L-prolylchloride (TPC) to form diastereoisomers followed by chromatography on an optically inactive stationary phase. TPC has been used for the resolution of numerous asymmetric amines, including ephedrine (73, 74, 75). TPC-derivative of ephedrine was prepared through addition of 0.1 M TPC solution in chloroform to the amine in chloroformic solution. After 10-15 min the solution was injected into a gas chromatograph with FID detector on a 3 % SE 30 packed column on Chromosorb G (AW, DMCS treated, 100-120 mesh), at 170°C oven temperature isotherm and 220°C injection block temperature using nitrogen (25 ml/min) as carrier gas. A quantitative determination of the enantionmetric percentages of (-) ephedrine, (+) and (-) norephedrine-TPC derivatives was possible. The method was found to be suitable for biological studies (76). Ephedrine was determined by treating the sample with sodium periodate to form benzaldehyde and gaschromatographing a solvent extract of this mixture. The oxidation step was included because ephedrine did not give a well-resolved peak (77).

A rapid GLC method for the determination of ephedrine hydrochloride in suspension formulation along with other components such as

theophylline and phenobarbital with α-naphthylamine as an internal standard is described. The analysis was performed on a 3 % OV 17 on Gaschrom Q 100-200 mesh packed column using a flame ionisation detector. The injection port, column and detector temperatures for the assay of ephedrine hydrochloride were maintained at 200, 150 and 200°C respectively. For the assay of ephedrine hydrochloride the sample was diluted with water, the pH was adjusted to pH 11 with 20 % NaOH and the solution was extracted twice with chloroform. The chloroform solution was injected directly into gas chromatograph. Several additional substances such as flavouring or colouring agents were extracted by chloroform along with ephedrine, but they did not interfere with ephedrine assay. The mean-recovery in the assay of synthetic mixtures for ephedrine hydrochloride was 99.2 $\pm$ 0.6 % and for commercial suspension 95.8 $\pm$ 0.8 % (78). A method for the assay of ephedrine hydrochloride is recommended by the joint committee of the pharmaceutical society and the society for analytical chemistry of Great Britain (79). The assay procedure is a modification of the method proposed by Beckett and Wilkinson (80). Ephedrine hydrochloride tablets, elixir, syrup and nasal drops have been analysed. An internal standard phendimetrazine bitartrate was added to the sample prior to its extraction. The aqueous solution is made alkaline with 20 % NaOH and the liberated ephedrine base is extracted with diethylether, the ether extract dried with anhydrous sodium sulphate, evaporated and made up to volume with ether. This solution is injected into gaschromatograph with a FID. The packing used in the 1 m, 4 mm i.d. glass column was 80-100 mesh, acid washed, silanised Chromosorb G impregnated with 2 % carbowax 6000 and 5 % of potassium hydroxide. The oven, injector and detector temperatures

maintained were 150, 200, 200°C, respectively with nitrogen as carrier gas (35 ml/min). The retention times for phendimetrazine bitartrate base and ephedrine were found to be 3 and 5 min respectively. The collaborative study carried on found this procedure adequate for the determination of ephedrine in certain pharmaceutical preparations. An examination by mass spectrometry and IR analysis of the ephedrine peak confirmed that ephedrine base was eluted intact. Some tailing of the ephedrine peak was noted by some collaborators. In general coefficients of variation within laboratories were not greater than 3 % (80). An electron-capture GLC procedure for determination of plasma ephedrine concentrations is described (81). The procedure is capable of determining 2 ng/ml of ephedrine. Pentane extraction of the drug and the internal standard 3,4-dimethoxyamphetamine and formation of the N-pentafluorobenzyl derivatives were followed by GLC determination. The analysis was performed on a 0.9 m x 2 mm i.d. glass column filled with 3 % OV 225 on Chromosorb W, AW-DMCS, 100-120 mesh. The injection port, oven and detector temperatures were 250, 235 and 325°C respectively. An electron-capture 63 Ni detector was operated with a standing current of 3.0 n amp. Argon-methan (95:5) as a carrier gas was maintained at 93 ml/min. The retention times of N-pentafluorobenzoyl derivatives of ephedrine and internal standard were reported to be 1.40 and 3.82 min respectively. Different GLC stationary phases such as OV-7, OV-17 were tried but were not suitable for quantitation. The drug and internal standard were poorly resolved with the former and broad peaks were obtained with the later. A OV-25 column gave sharp peaks for ephedrine and internal standard derivatives, but the norephedrine, the major metabolite of ephedrine, could not be separated. Formation of N-trifluoroacetyl, N-pentafluoropropionyl,

N-heptafluorobutryl and N-pentafluorbenzoyl derivatives and their glc-mass spectrometric identification are discussed together with comparative electron-capture sensitivities of these derivatives with Nickel-63 detector. The detection of the N-pentafluorobenzoyl derivative of ephedrine is at least 100-fold greater in sensitivity than detection of the N-trifluoroacetyl derivative (81). Heptafluorobutyryl ephedrine derivatives following benzene extraction of alkaline serum were used for electron-capture analysis of blood levels at therapeutic dosages (82). The procedures reported earlier were found insufficiently sensitive for clinical use (83). The determination of ephedrine plasma levels were performed through GLC with FID using a 8 % carbowax 20 M + 2 % KOH on Chromosorb W column. Ephedrine was extracted from plasma with diethylether after alkalising the sample with NH_4OH (84). GLC with FID determination of ephedrine was performed on a 2.0 % Carbowax 20 M and 5 % KOH on Chromosorb G (100-120 mesh), AW, DMCS column at oven temperature of 100°C (22). Gas chromatography has been further applied for the specific quantitative determination of ephedrine and its metabolite norephedrine in urine (85, 86). A 1.83 m, 4 mm i.d. 3 % OV 1 on Gaschrom Q, 100-200 mesh column at 140°C oven, 140° injection block and 230°C detector temperatures was used to monitor urinary excretion of ephedrine in man through gas chromatography with FID (87). Derivatives of ephedrine with trialkylsilyl groups attached to the hydroxyl function of the molecule were synthesised and tested gas chromatographically on a 2 m 3 % OV 17 on chromosorb G, AW, DMCS glass column at oven temperatures between 175-185°C (88). N-TFA-L-alanine and N-TFA-L-alanyl chloride were used for the preparation of high volatile diastereomeric derivatives of (-) and (+)-ephedrine which were separated along with

the derivatives of other chiral amino alcohols and amines on a 30 m glass capillary column OV-17 or SE 30 at a column temperature of 200°C with FID (89). The separation of the N,O-pentafluoropropionyl derivatives of the enantiomers of ephedrine and of some analogues has been carried out using chirasil-val (90). N,O-Bis-heptafluorobutyryl derivatives of ephedrine and other analogues were separated on a 18-m glass capillary column coated with XE-60-L-valine-(R)- α-phenylethylamide (91). Excellent separations of diastereomeric derivatives of several amino alcohols of the ephedrine type have been obtained on a column with a chiral stationary phase after N-acylation with L- α-chloroisovaeryl chloride and o-trimethylsilylation (92). König and Benecke reported the resolution of a number of amino alcohols including the N-demethylated analogues of ephedrine and norephedrine on a GLC chiral modified OV-225 phase (93).

High Performance Liquid Chromatography

The separation and quantitation of ephedrine is carried out by means of HPLC after its conversion into 4-nitrobenzamide with 4-nitrobenzoyl chloride. A procedure for the preparation of derivatives is given. The determination was carried out on a 20 cm spherisorb 5 μm column using a solvent mixture of isooctane methylene chloride + ethanol + water (400+87+8+5) as a mobile phase with 1.8 ml/min flow-rate and the ephedrine derivative was detected at 337 nm. The detection limit is given as about 5 ng per 20 μl injected pure derivative with a relative standard deviation of less than ± 6 %. The method could also be applied for the analysis of plasma samples (94). The quantitative determination of combination of antihistamine and decongestant drugs inclu-

ding phenylepherine, dl-ephedrine, l-ephedrine, chlorpheniramine etc. contained in solid and liquid dosage forms are described. All active ingredients except the ephedrine optical isomers were separated from other ingredient with ion-paired HPLC. Elixirs, syrups, tablets and timed-release capsules or tablets were analysed. The chromatographic separation was done on a 4 mm i.d. x 30 cm μ-Bondapak phenyl column with a mobile phase water-methanol-glacial acetic acid (55+44+1 V/V) containing enough heptanesulfonic acid sodium salt to yield a 0.005 M solution. The flow rate was 2.0 ml/min and the detection wavelength 254 nm. The column used is capable of resolving almost all of the compounds except the stereoisomers l-ephedrine and d-ephedrine. The reproducibility of the method was excellent with a coefficient of variation of 0.9 % (95). Simultaneous determination of ephedrine sulphate, hydroxyzine hydrochloride and theophylline in tablets by HPLC involved a 10 μm Bondapak C_{18} column with acetonitrile-aqueous ammonium carbonate solution (50+50) at pH 7.0 as the eluent and UV detection at 254 nm. 0.1 % aqueous ammonium carbonate buffer was prepared and adjusted to pH 7.0 with acetic acid (96). The chiral forms of ephedrine were analysed as the corresponding oxazolidines formed by reaction between the propanolamine and 2-naphthaldehyde. HPLC separation was carried out on a column of 25 cm x 4.6 mm i.d., 5 μm aminopropyl-bonded silica gel modified with (R)-N-(3,5dinitrobenzoyl)phenylglycine using a mobile phase n-hexane-isopropanol (99.5+0.5) with a flow rate 1.0 ml/min at 254 nm (97). Following their conversion to dithiocarbamate ligands and subsequently to nickel complexes, the separation and quantitation of enantiomeric mixtures of ephedrine and pseudoephedrine were accomplished by liquid chromatography with

ternary solvent mixtures. Formation of nickel complexes prior to chromatography and on-column formation using nickel(II)ions in the mobile phase has been studied (98). Ephedrine and pseudoephedrine in formulated products were determined on a 10 µm alkylphenyl µ-Bondapak column with acetonitrite-water-monobasic sodium phosphate (1 % acetonitrile in 0.05 M monobasic sodium phosphate aqueous solution) as the eluent and UV detection at 210 nm (99). A HPLC method is described in which ephedrine hydrochloride is measured after its oxidation to benzaldehyde through periodate simultaneously with theophylline and phenobarbital in tablets with butabarbital as the internal standard. A pH of 7.8 was selected for rapid oxidation of ephedrine and a detection wavelength of 241 nm was chosen which is near to the maximum for benzaldehyde and barbiturates and to the minimum for theophylline. Chromatographic column was a reversed phase C_{18} phase bonded on silica and the mobile phase consisted of acetonitrile (240 ml) mixed with 0.01 M phosphate buffer, pH 7.8 (760 ml). Benzaldehyde obtained from ephedrine had a retention time of 11.7 min. The chromatogram showed no interference from the excipients and other oxidation products. Procedures are provided for the assay of conventional and sustained-action tablet formulations (100). In another HPLC method the simultaneous assay of ephedrine hydrochloride, theophylline and phenobarbital in tablets is reported. 25 cm x 4.5 mm i.d., 10 µm Partisil ODS II column and a methanol-0.007 M monobasic potassium phosphate (37+63, pH 2.3) as mobile phase was used at detection wavelength 254 nm. A methanolic extract of the powdered sample containing salicylamide as the internal standard was injected into the chromatograph (101). In the determination of ephedrine using reversed-phase ion-pair liquid chromatography, a chromatographically pure sample

was observed to give three peaks under certain mobile phase condition. The peak due to ephedrine was found to vary from symmetrical to almost completely resolved split peaks in mobile phases containing only PIC B_7 (heptane sulfonic acid). When sodium sulphate was included in the mobile phase peak-splitting was more pronounced. A proposal, that peak splitting was the result of the composite interplay of two discrete chromatographic mechanism, was investigated. The results of analysis by GC/MS confirmed that each peak was due to ephedrine, however, only one of the three split peaks was found to contain ion-pairs. It is postulated that peak splitting is a physical phenomenon on reversed-phase column and the separation of these drugs by ion-pair HPLC is based on a mixed rather than a single mechanism (102). Derivatization of ephedrine with dansyl chloride and a sensitive, specific HPLC method for its determination in complex pharmaceutical dosage forms is reported. A 25 cm x 2.8 µm i.d. column filled with Merck silica gel SI 100, 10 µm and diisopropyl ether saturated with conc. Ammonia-isopropanol (99+1) were used for separation. The detection was carried out simultaneously with a fluorescence detector, 354 nm excitation, 476 nm emission, and a fixed-wavelength 254 nm UV detector (56). Determination of ephedrine sulfate in cough-cold mixtures along with various other analgesic and antihistamine compounds was performed on a Corasil C_{18} column with the mobile phase acetonitrile-water (60:40) with 1 % ammonium acetate and the pH adjusted to 7.40 (103).

HPLC retention characteristics of ephedrine have been measured along with 84 other basic drugs of forensic interest. Chromatography was carried on using 250 x 5 mm i.d. column packed with Spherisorb S5W at 254 nm. The

eluent consisted of methanol-aqueous ammonium nitrate buffer (9+1). The buffer was prepared by adding 94 ml ammonia (35 %) and 21.5 ml nitric acid (70 %) to 884 ml water and then adjusting the pH to 10.1 with ammonia. The flow-rate was 2 ml/min. Because of the alkaline nature of the eluent, a short column, dry packed with silica (40 µm) was included between the pump and injector to minimise dissolution of the analytical packing material (104).

Resolution of the enantiomers of ephedrine and other related compounds through a simple HPLC method is described. A 150 mm x 4.6 mm column was packed with Ultrasphere ODS, 5 µm particle size and the mobile phase was prepared by mixing 400 ml acetonitrile with 600 ml water containing 1.4 g of monobasic ammonium phosphate. The flow-rate was 1.0 ml/min and the column eluent was monitored at 254 nm. Ephedrine or ephedrine hydrochloride is derivatised with the chiral reagent 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and the separation of the resulting diastereomeric thioureas is performed by reversed phase HPLC. The derivatisation method is extremely simple, the chiral reagent is commercially available, chemically and stereochemically stable. The resolution of ephedrine, pseudoephedrine and norephedrine is considerably better (105). R-α-Methylbenzyl isothiocyanate, a commercially available chiral compound, was evaluated as a chiral derivatizing agent for the separation of among others ephedrine's enantiomers through HPLC on a 150 x 4.6 mm Ultrasphere ODS 5 µm column with a water-acetonitrile (50:50) mobile phase at 1.0 ml/min flow-rate (106).

The enantiomers of ephedrine were resolved as their cyclic oxazolidine derivatives which

were produced by the condensation of the amino alcohol with 2-naphthaldehyde. The enantiomeric resolution of ephedrine was performed on an ionically bonded chiral stationary phase. The column was a stainless steel Regis-packed pirkle type 1-A (250 x 4.6 mm) with an α-aminopropyl packing of 5 μm spherical particles modified with (R)-N-(3,5-dinitrobenzoyl)phenyl-glycine. The mobile phase consisted of hexane-isopropanol (99.5+0.5), the flow-rate was 1.0 ml/min and the detection wavelength was set at 254 nm (107).

Ephedrine is converted to metal (copper and nickel) dithiocarbamate complexes by means of a pre-column derivatisation method. Chromatography is done on a Lichrosorb RP 18 column with mixtures of acetate buffer (pH 5.8) and organic solvents like methanol, acetonitrile or ethanol as mobile phases. The complexes were detected amperometrically (applied potential + 0.7 V VS SCE) using a thin-layer electrolytic cell fitted with glassy carbon working and auxiliary electrodes. The spectrophotometric detector was set at 325 nm for nickel chelates and at 270 nm for copper chelates. The procedure is described to have a great sensitivity (about 10^{-12} M) and good selectivity for the more substituted amino drugs (108).

9.5. NMR-Assay

A rapid NMR method is described for the determination of ephedrine hydrochloride in tablets. The NMR spectrum of ephedrine hydrochloride in D_2O displays chemical shifts for methyl proton doublet at 1.16 ppm, N-methyl proton singlet at 2.83 ppm and aromatic protons singlet at 7.43 ppm. The doublet at 1.16 ppm was chosen for quantitative work. Acetamide was used as an internal standard on

the basis of its methyl three proton singlet at 2 ppm, which is sufficiently separated from the 1.16 ppm doublet of ephedrine hydrochloride and the solvent signal at 4.75 ppm to allow satisfactory determination. The results for synthetic mixtures and tablets are comparable to those obtained by other methods. The relative standard deviations were 0.5 and 1.2 % for the pure drug and tablets respectively. In addition the NMR method furnishes a specific means of identification of ephedrine (109).

9.6. Radioimmunoassay

Stereospecific radioimmunoassay for 1-ephedrine and d-ephedrine in human plasma after administration of a single 50 mg oral dose of dl-ephedrine hydrochloride has been reported (110). Separate RIAS developed for d-ephedrine and l-ephedrine were used to measure the concentrations of the enantiomers of ephedrine in the blood of two volunteers dosed with racemic ephedrine. The RIAS were validated by comparing the sum of the concentrations of the enantiomers with total ephedrine concentrations determined by a nonstereoselective GLC-ECD method. Fig. 9 shows graphically plasma concentrations of d- and l-ephedrine (110). Budd has presented a comparison of GC and EMIT (enzyme multiplied immunoassay technique) methods for the analysis of ephedrine and other related drugs. For GC analysis an Apiezon-KOH column was used (111).

9.7. Isotachophoresis

The utility of isotachophoresis has been examined for the simultaneous determination of all the six ephedrine alkaloids in ephedra

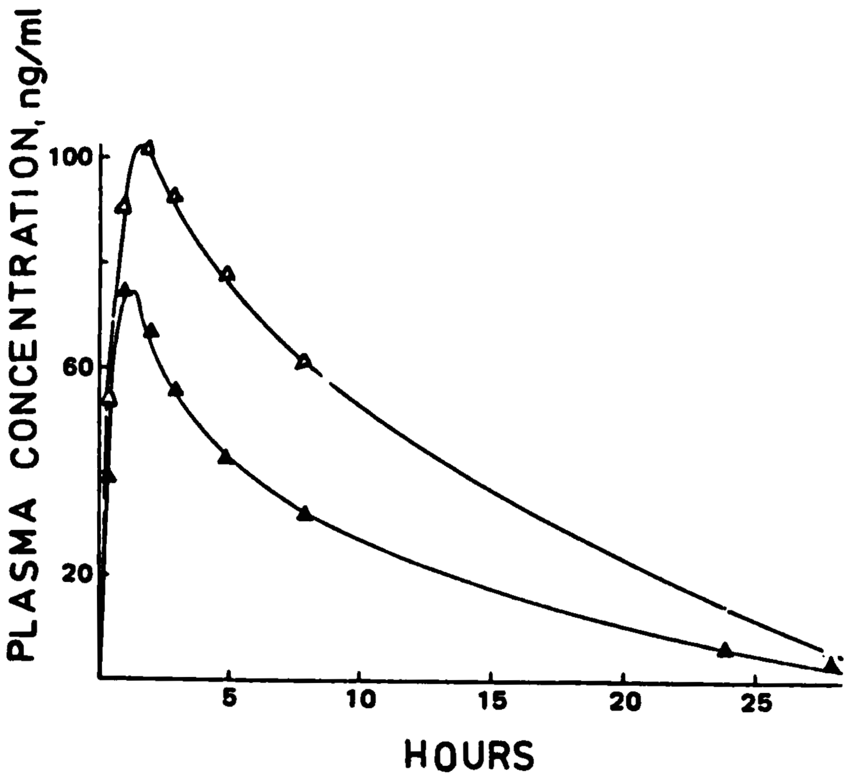


FIG. 9

D-EPHEDRINE (Δ) AND L-EPHEDRINE (▲) CONCENTRATIONS IN THE PLASMA OF A HEALTHY HUMAN VOLUNTEER FOLLOWING A SINGLE 50 MG ORAL DOSE OF DL-EPHEDRINE HYDROCHLORIDE (110)

herb extracts. Ephedra herb was extracted with 50 % ethanol. All analysis were carried out on a Shimadzu model Ip-2A Isotachophoretic analyzer equipped with a Shimadzu potential gradient detector with a 40 mm PTFE capillary (1.0 mm i.d.) as the pre-column and a 150 mm FEP capillary (0.5 mm i.d.) connected in series. The leading electrolyte was prepared by adding β-alanine or histidine to 0.005 M barium hydroxide to adjust the pH. 0.1 % Triton X-100 was added to improve separation by enhancement of viscosity and reduction of electrodenosmosis. 0.01 M Ammediol was used as the terminating electrolyte. The analysis was first proceeded at 200 μA for 12 min and was then continued at 100 μA. Calibration curves for the six ephedrine alkaloids were constructed in the range of 0.2 - 2.0 mg/ml. The ephedra extract (20.0 mg) was dissolved in water (1.0 ml) and an aliquot (0.010 ml) was injected directly (112).

10. Drug Metabolism and Pharmacokinetics

The uses of ephedrine indicate that the drug has sympathomimetic actions grossly resembling those of epinephrine. It is readily absorbed from the upper gastrointestinal tract. Good effects have also been achieved from the lower tract also through its employment in a retention enema. The gastrointestinal absorbability and the long duration of action has been shown to be associated with the attachment of a methyl group on the alpha carbon. Ephedrine is not oxidized by enzymatic action, but conjugation occurs in some species (113). Ephedrine is readily and completely absorbed from the GI tract, peak plasma concentrations being achieved about an hour after oral administration. It is resistant to metabolism by

monoamine oxidase and is largely excreted unchanged in the urine, with some deaminated and N-demethylated metabolites. Half-life lies between 3-6 h. Elimination is enhanced and half-life is accordingly shorter in acid urine (114). The relative metabolism and urinary excretion of the ephedrines are dependent upon the urinary pH and in certain circumstances the volume also (115). Intra-subject variation was observed in both the lag time and the rate constant for absorption in case of (-)ephedrine. Ephedrines were readily absorbed within 3 hours of administration. Mean overall elimination t 1/2 value for unchanged (-)ephedrine was 3.03 h. It appears that ephedrine and norephedrine, when present in the body as metabolites, are eliminated faster than when they are administered per se. A possible explanation for this phenomenon is that the kinetics of elimination of the ephedrines is dose-dependent; elimination is faster at low body drug levels than at high levels. The kinetics of absorption, metabolism and excretion of (-)-ephedrine has been elucidated using analog computer analysis of urinary excretion data from three male subjects under constant acidic urine control (116). In a feasibility study 25 mg single oral doses of ephedrine sulfate in the form of a commercial syrup and two commercial capsules were administered in crossover fashion to three non-smoking subjects. Urinary pH was not controlled, but adequate urinary flow rates were maintained by regulated water intake. There were no significant differences in average amounts of ephedrine excreted during any of the 11 sampling intervals, in average peak excretion rates, nor in average times of occurrence of the peak excretion rate. The average elimination half-life of ephedrine was 5.99 h and average urinary pH 6.30. The urinary excretion data was adequately described by the two-compartment open model with first-order

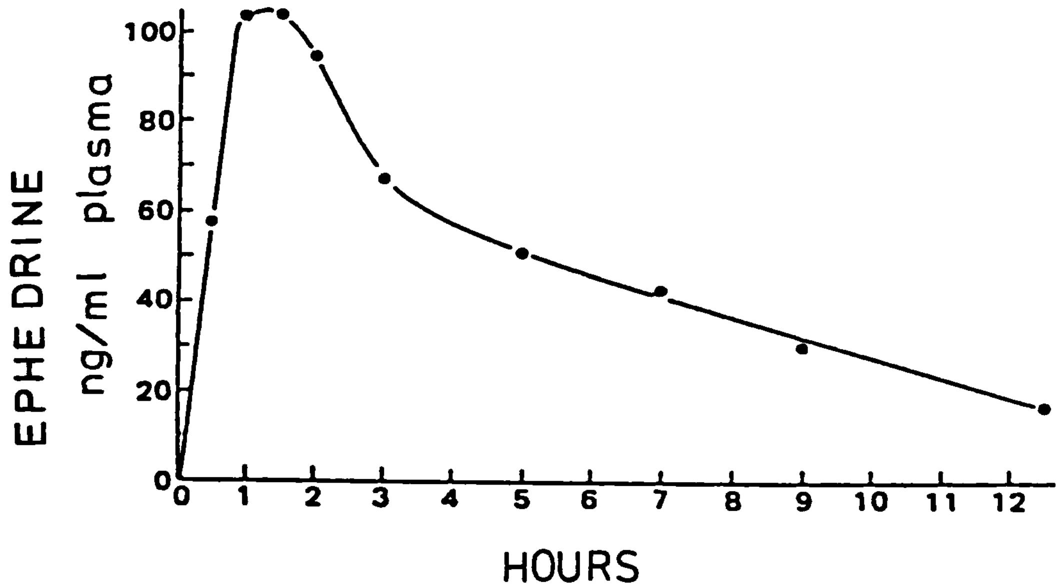


FIG. 10

EPHEDRINE CONCENTRATIONS IN THE PLASMA OF A HUMAN VOLUNTEER (67.6 KG) FOLLOWING A SINGLE 24 MG DOSE OF EPHEDRINE HYDROCHLORIDE IN A COMBINATION TABLET (81)

absorption and lag time. The baseline concentration of ephedrine in plasma 15 hours after the night dose was 20 ng/ml. Following administration of one tablet containing 15 mg ephedrine sulphate the concentration rose to 95 ng/ml after 4 h, falling to 65 ng/ml at 6 h (84). Ephedrine is readily absorbed after oral or percutaneous administration; gastrointestinal absorption is increased by antacids but decreased by kaolin. After an oral dose of 22 mg of ephedrine hydrochloride plasma concentrations of 40-140 ng/ml are obtained and after an oral dose of 33 mg peak plasma concentrations of 65 - 120 ng/ml are attained during therapy: effective bronchodilator plasma concentrations are in the range of 35 - 80 ng/ml and plasma half-life lies between 3 - 11 hours. Metabolic reactions are N-demethylation and oxidative deamination followed by conjugation. Upto about 95 % of a dose may be excreted in the urine in 24 hours, 55 - 75 % as unchanged drug, 8 - 20 % as the N-demethylated metabolite and 4 - 13 % as deaminated metabolites such as benzoic acid, hippuric acid and 1-phenylpropane-1,2-diol. The rate of urinary excretion of ephedrine is pH-dependent and is increased in acidic urine (117). A combination tablet containing ephedrine hydrochloride (24 mg) theophylline (130 mg) and phenobarbital (8 mg) was administrated and aliquots of plasmas collected showed peak plasma concentration of over 100 ng/ml. Plasma concentrations over 24 h in the subject fit a one-compartment model from which the elimination half-life was calculated as 4.8 h. Fig. 10 graphically illustrates this phenomenon (81).

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ESTRADIOL

Eugene G. Salole

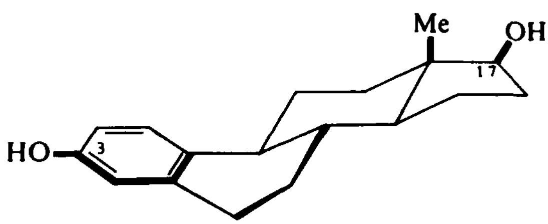
1. Foreword

Estradiol is the most potent estrogenic hormone, secreted in normal pre-menopausal women mainly by the ovaries. The last of the major natural hormones to be isolated (12mg were extracted from 4ton of porcine ovaries in 1935, although it had previously been synthesized from estrone), its main clinical use is in post-climacteric replacement therapy, for which it is administered (alone and in combination with other hormones) by the oral, transdermal, subcutaneous and intravaginal routes in a variety of dosage forms[1,2].

The intense interest in estrogens since the turn of the century has generated a vast, and still rapidly expanding, literature; this modest profile therefore aims principally to collate some of the physicochemical data on estradiol which would be of interest to pharmaceutical scientists. Reviews of the biochemistry, molecular pharmacology and other biological aspects of estradiol may be found in the books edited by Chaudhury[3] and Makin[4]; its chemistry is covered by Fieser and Fieser[5] and in Beilstein's Handbook[6].

2. Description

Estra-1,3,5(10)-triene-3,17β-diol; 17β-estradiol; E_2; obsolete terms include dihydrofollicular hormone, dihydrofolliculin, dihydrotheelin and dihydroxyestrin ('17α-estradiol' is a misnomer in the literature prior to ca. 1952); Chemical Abstracts Service registry number 50-28-2.



$C_{18}H_{24}O_2$

$\underline{M}_r$ 272.38$_7$

(see Sect. 4.7)

A white, odorless, tasteless, crystalline powder.

3. Synthesis

The total synthesis of estradiol from acyclic precursors has been accomplished[6,7], but it is usually prepared by the sodium borohydride reduction of estrone, itself obtained by the pyrolysis or reductive aromatization of androstenedione[8]. Syntheses of isotopically-labelled estradiol have been described[9].

4. Physical Properties

4.1 Infrared Spectrum

The IR spectrum of estradiol hemihydrate is illustrated in Fig. 1; detailed assignments have been proposed by Smakula et al[10].

The spectrum in Fig. 1 is identical to that reported by Neudert and Ropke[11] and Hayden et al[12]., and to one supplied by the MRC Steroid Reference Collection, London, but quite different from the spectra in the Sadtler and Sammul et al., compilations[13], which also differ from each other (see Sect. 4.7).

4.2 Nuclear Magnetic Resonance Spectra

^1H- and ^{13}C-NMR spectra of estradiol hemihydrate in acetone-d_6 were obtained at 250.13 and 62.9MHz, respectively. Assignments for the proton spectrum (Fig. 2) are:

δ/ppm	
7.09	(IH, d, J = 8.3Hz, H-1)
6.59	(IH, dd, J = 8.3, 2.8Hz, H-2)
6.52	(IH, d, J = 2.8Hz, H-4)
4.10	(3H, br.s, 2xOH & 1/2 H_2O)
3.67	(IH, dd, J = 8.8, 8.1Hz, H-17α)
2.76	(2H, m)
2.28	(IH, m)
2.20-1.10	(12H, m)
0.77	(3H, s, Me-18).

The following assignments for the ^{13}C-NMR spectrum (Fig. 3) concur with the literature[14]:

δ/ppm		δ/ppm	
155.89	(s, C-3)	44.04	(s, C-13)
138.38	(s, C-5)	40.03	(d, C-8)

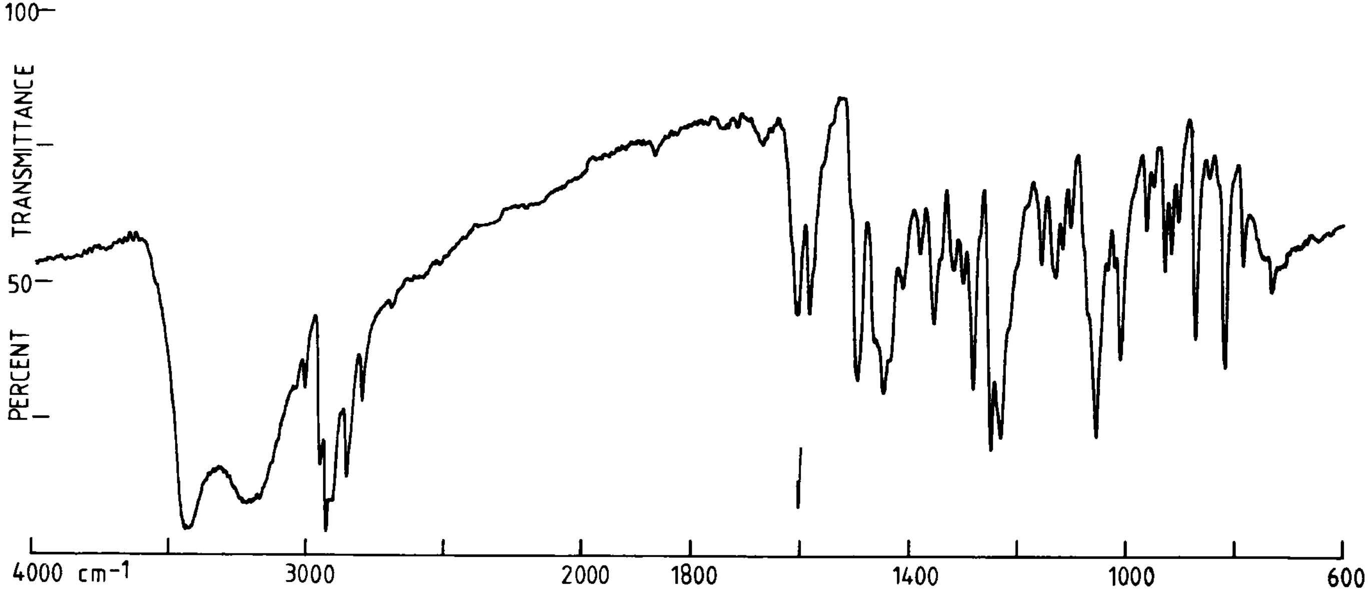


Fig. 1 IR spectrum of estradiol hemihydrate: Pye-Unicam SP3-200 spectrometer, KCl disc (polystyrene marker at 1602cm^{-1}).

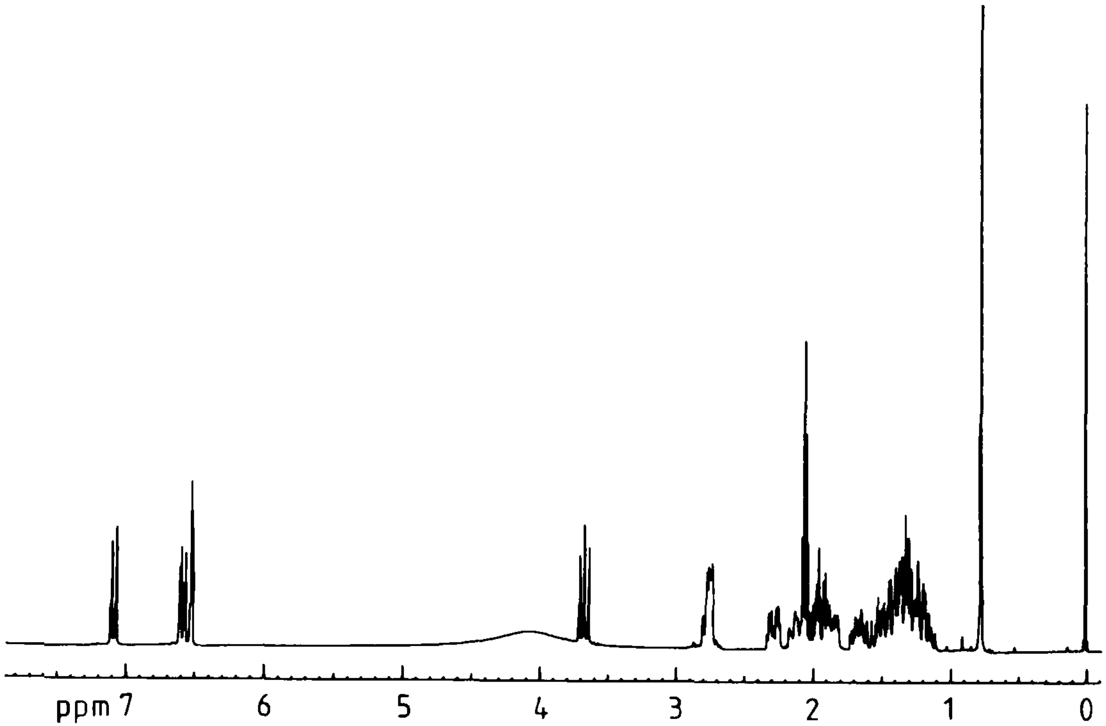


Fig. 2 ^{1}H-NMR spectrum of estradiol hemihydrate: Bruker WM250 spectrometer at 250.13MHz, acetone-d_6 solution, TMS internal standard.

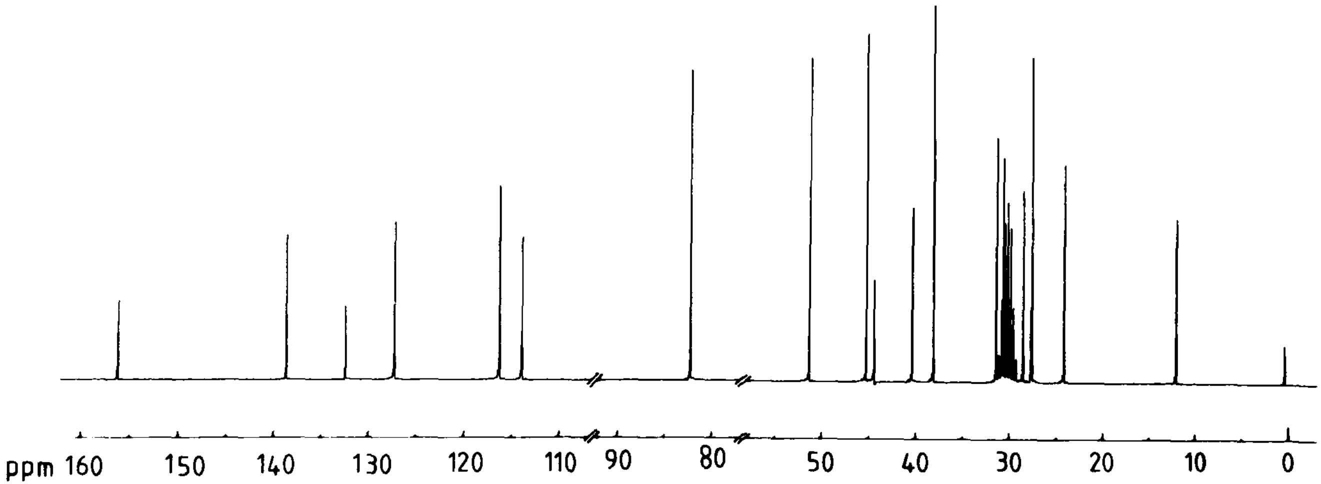


Fig. 3 ^{13}C-NMR spectrum of estradiol hemihydrate: Bruker WM250 spectrometer at 62.9MHz, acetone-d_6 solution, TMS internal standard.

132.09	(s, C-10)	37.76	(t, C-12)
126.94	(d, C-1)	31.02	(t, C-16)
115.96	(d, C-4)	30.30	(t, C-6)
113.58	(d, C-2)	28.13	(t, C-7)
81.86	(d, C-17)	27.26	(t, C-11)
50.96	(d, C-14)	23.80	(t, C-15)
44.90	(d, C-9)	11.63	(q, C-18).

4.3 Ultraviolet Spectrum

In 2%v/v methanol, estradiol hemihydrate exhibits UV maxima at 221nm (A1%1cm 289) and 280nm (A1%1cm 75), with a shoulder at 287nm (Fig. 4). Absorbance values quoted for estradiol in other media are:

	λmax/nm	A1%1cm
0.1M hydrochloric acid[15]	278	76
80%v/v methanol, alkalinified[11]	285.6	69
0.1M sodium hydroxide[15]	238	341
	296	102
6M ammonia[16]	240	309
	297	98.

The absorption spectrum of estradiol undergoes bathochromic shifts with rise in pH (isosbestic point at 285.6nm[11]); spectra in concentrated sulfuric acid are illustrated in Ref. 17.

4.4 Mass Spectrum

The following assignments of a high-resolution mass spectrum, obtained using an AEI (Kratos) MS9 spectrometer (electron impact ionisation, direct insertion) agree to six significant figures with the experimental mass data for estradiol hemihydrate:

m/z	Composition	Rel.Int.
272	$C_{18}H_{24}O_2$ [M^+]	100
213	$C_{15}H_{17}O$	46
186	$C_{13}H_{14}O$	14
185	$C_{13}H_{13}O$	13
172	$C_{12}H_{12}O$	27
160	$C_{11}H_{12}O$	43
159	$C_{11}H_{11}O$	29
158	$C_{11}H_{10}O$	13
146	$C_{10}H_{10}O$	28
145	$C_{10}H_9O$	18
133	C_9H_9O	23.

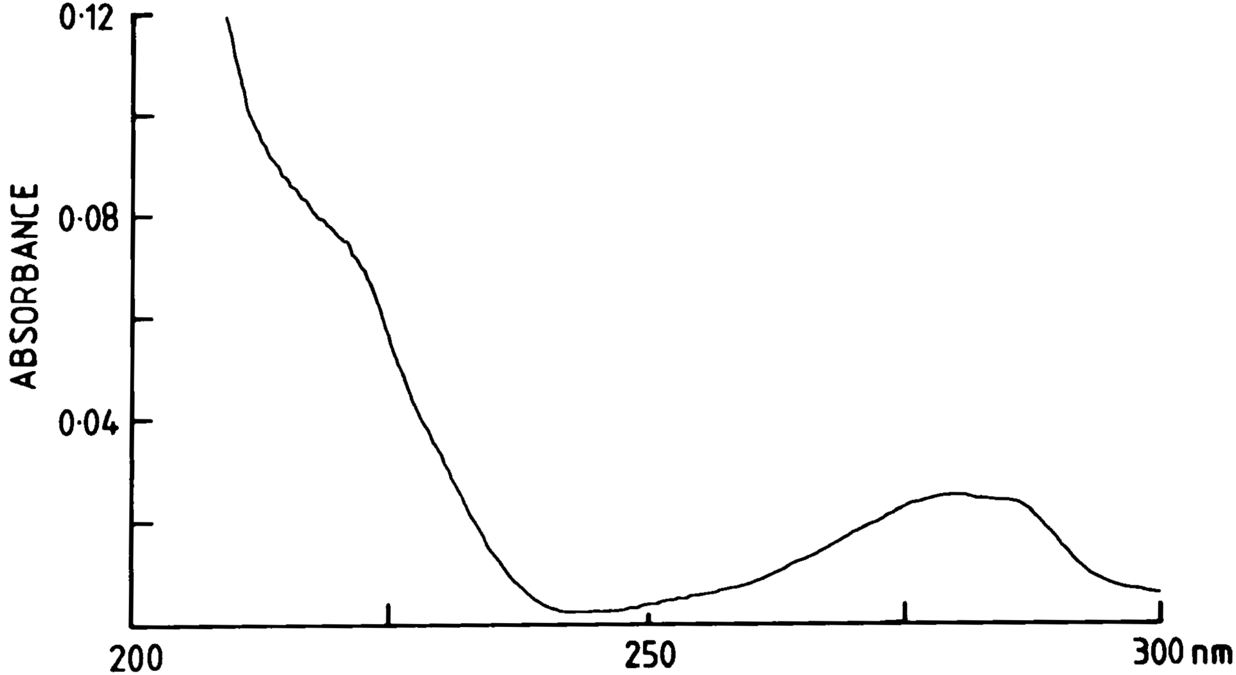


Fig. 4 UV spectrum of estradiol hemihydrate: Cecil CE505 spectrophotometer, 0.33mg dl^{-1} in 2.0%v/v methanol.

A simulated low-resolution plot of the spectrum is illustrated in Fig. 5. The chemical ionization mass spectrum has been described[18].

4.5 Dipole Moment, Optical Rotation and pKa

The dipole moment of anhydrous estradiol in dioxane was measured at 2.33D (7.772×10^{-30} Cm)[11].

Specific optical rotation values reported are:

$[\alpha]_D$ (22-24°C, dioxane) +76°[11]
(18°C, ethanol) +78°[19].

Values at different wavelengths and temperatures, and in other solvents, are quoted in Beilstein[6].

Spectrophotometrically determined apparent pKa values for the phenolic OH are :

10.12 ± 0.025 SD[20]
10.30 ± 0.10 MSE[21]
10.71 ± 0.02 SD[16].

4.6 Solubility, Complexation and Distribution Ratios

The solubilities of estradiol in some aqueous and organic media are listed in Tables 1-3. These values, particularly for aqueous solutions, should be accepted cautiously, because the apparent solubility of estradiol depends upon the determining procedure. For instance, the equilibrium concentrations achieved by unsaturated solutions may differ from those attained by initially supersaturated solutions[26], and whereas shaking an aqueous suspension for 48h resulted in solubility of 0.319mg dl^{-1} at 25°C, ultra-sonication for 0.5h instead produced a concentration of 0.613mg dl^{-1} [28]; the filters used to clarify supernatants may adsorb estradiol to a degree dependent on its concentration and their composition[29].

Although the 25mg dl^{-1} concentration rapidly achieved by a 1:9 solid co-precipitate of estradiol in polyvinylpyrrolidone 40000 was attributed to the steroid being in molecular dispersion[25], the aqueous solubility of estradiol may be modified by association with a variety of compounds. The amino acids tyrosine (Table 1), arginine and lysine

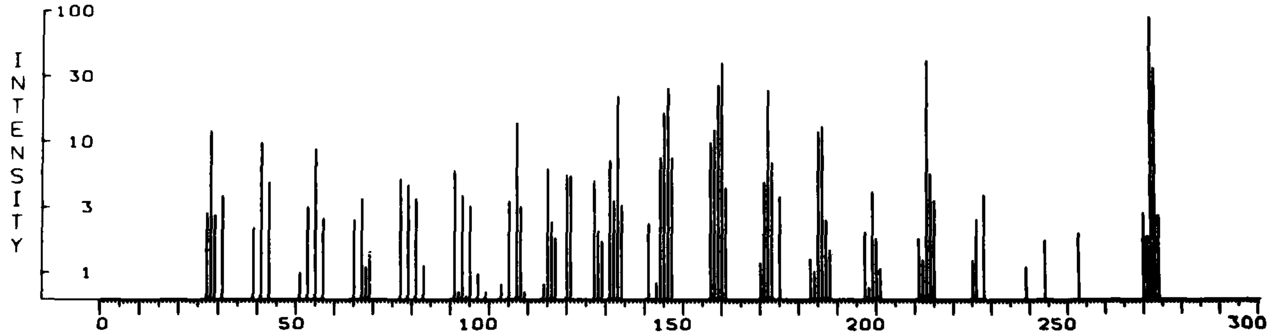


Fig. 5 Simulated (low-resolution) mass spectrum of estradiol hemihydrate.

TABLE 1

Solubility (mg dl^{-1}) of Estradiol in Aqueous Solvents

	Temperature/°C						
	20	25	35	37	42.5	50	Ref.
Water			0.399				22
	0.17	0.30	0.56		0.77	0.97	23
0.02 M Sodium chloride				0.38			24
0.20 M Sodium chloride				0.34			
0.40 M Sodium chloride				0.28			
Phosphate buffer,							
pH7.2, 0.05 I				0.38 ± 0.046			
pH7.2, 0.10 I				0.36 ± 0.016			
pH7.2, 0.20 I				0.34 ± 0.024			
0.005 M L-tyrosine in phosphate buffer, pH7.2, 0.10 I				0.66 ± 0.049			
Phosphate buffer, pH7.4, 0.15 I				0.512			25
0.02 M Sodium deoxycholate in phosphate buffer, pH 7.4, 0.15 I				3.92			

TABLE 2

Solubility (mg dl^{-1}) of Estradiol in Organic Solvents[26]

	Temperature/°C				
	0	15	25	30	40
Acetone	2594.1	4291.2	7068.8	8914.8	13715.2
Benzene	-	21.7	46.7	80.1	198.3
Chloroform	140.5	251.2	411.4	642.7	762.0
Cyclohexane	-	0.5	1.3	2.2	7.6
Dichloromethane	45.1	108.4	192.6	267.1	-
Dioxane	-	7075.2	12085.6	19868.4	30968.0
Ethanol	1541.8	2387.2	3134.4	3727.4	4890.7
95% v/v Ethanol	1060.4	1606.9	2908.8	4186.3	4805.3
Diethyl ether	533.2	700.8	754.5	836.7	-
Hexane	< 0.5	< 0.5	< 0.5	0.9	-
Methanol	862.1	1811.3	2548.8	3525.6	5342.4
Tetrahydrofuran	25447.2	28006.4	29222.4	33788.8	43313.6
Toluene	4.3	14.8	33.1	51.5	95.0

TABLE 3

Solubility (mg g^{-1}) of Estradiol in Organic Solvents at 22°C[27]

1-Decanol	28
Dimethyl sulfoxide	> 500
Ethyl oleate	16
Ethylene glycol	16
Ethylene glycol: polyethylene glycol 400, 1:1 w/w	78
Glycerol	1.5
Polyethylene glycol 400	105*
Polysorbate 80 (Tween 80)	36
Propylene glycol	75
80% w/w Propylene glycol	2.8
60% w/w Propylene glycol	0.52
40% w/w Propylene glycol	0.10
20% w/w Propylene glycol	0.023
Propylene glycol: glycerol, 1:1 w/w	25

* 167×10^2 mgdl^{-1} at 35°C[22]

promote solubility[24], as do progesterone[29] and human serum albumin[30] (which admixed with propylene glycol has been used to prepare intravenous injections of 100mg dl^{-1} [31]). Polysorbate 20 (at 20°C) and sodium dodecyl sulfate (at 40°C) maximally solubilized estradiol at 13 and 25mmol per mol surfactant, respectively[32]; with egg lecithin vesicles the ratio was found to change from 0.0176 to 0.0422mmol per mol lipid depending on their method of preparation[28]. β-cyclodextrin was found to complex with estradiol in aqueous solution; the white amorphous powder isolated by interfacial co-precipitation exhibited a rapid rate of dissolution, with a solubility at 25°C of 12mg dl^{-1} [33]. In contrast, both urea and digitonin form only sparingly soluble complexes with estradiol: columnar crystals (of orthorhombic symmetry) of the 1:1 complex precipitate from a solution in benzene of estradiol and urea in 1:10 mole ratio[34], whereas needles of the digitonide complex (m.p.≈265°C) may be obtained by mixing solutions of the steroid with 1-4%^{w}/v digitonin in 80%^{v}/v ethanol[35]. Apparently the urea complex is not of the clathrate type[34], and like the digitonide is readily cleaved (by warming in water and dry pyridine, respectively).

Estradiol is sufficiently soluble in peanut and sesame oils for them to be used as vehicles for intramuscular injections[36].

Distribution ratios of estradiol (and its esters) in several dozen systems have been compiled by Engel[17]; a selection of values at room temperature are listed in Table 4. Lundberg[23] has determined the thermodynamic parameters associated with the partitioning of estradiol between octanol and water.

TABLE 4

Distribution Ratios of Estradiol[17]

Solvent system	D_c
Benzene/Water	∞
Benzene/1.54M Hydrochloric acid	∞
Benzene/0.10M Sodium hydroxide	0.23
Benzene/1.0M Sodium hydroxide	0.04
Benzene:petroleum ether, 1:1/Water	24
50%^{v}/v Methanol/Carbon tetrachloride	2.10
Water/Carbon tetrachloride	0.08

Diethyl ether/Water	55
Diethyl ether/1.6M Hydrochloric acid	50
Diethyl ether/0.10M Sodium hydroxide	2.0
Diethyl ether/1.0M Sodium hydroxide	0.7
Ethyl acetate/Water	28
Hexane/Water	1.07
Petroleum ether (35-60°)/Water	0.79

4.7 Crystal Properties

Estradiol exhibits a variety of solid-state phases and transformations, which has led to a degree of confusion in the somewhat fragmented literature, e.g. different IR spectra in reference works (Sect. 4.1). This section attempts to put the crystal properties of estradiol into perspective by presenting a resume of published work, some of which is reinterpreted in the light of a close comparison of data and some unpublished results.

The most noteworthy property of estradiol is its tendency to adopt the hemihydrated form, in which phase it crystallises from not only partially aqueous solutions but also from ethyl acetate[10], chloroform[12], absolute ethanol[25,37] and other apparently anhydrous solvents[38]. In ignorance of this characteristic some crystallographic data has been erroneously ascribed, e.g. the X-ray powder diffraction data of Parsons and Beher[39] (Table 5), cited in the Joint Committee on Powder Diffraction Standards File, properly refers to estradiol hemihydrate, similarly the 'anhydrous' single crystal analysis reported by Norton et al[40]., and the 'monohydrate' data cited in Structure Reports[41] are erroneous.

TABLE 5

X-ray Powder Diffraction Data for Estradiol Hemihydrate[39]

d/Å	I/Io	d/Å	I/Io
7.50	4	2.90	2
6.71	9	2.80	4
6.03	4	2.65	1
5.64	10*	2.56	2
5.00	4	2.49	4
4.78	9	2.40	2
4.63	4	2.32	1

4.32	6	2.27	2
4.08	4	2.21	2
3.92	6	2.14	4
3.72	5	2.08	2
3.35	6	2.00	2
3.24	3	1.93	3
3.13	3	1.86	2
3.02	3	1.79	2

* These data were obtained by visual analysis of Debye-Scherrer photographs; the diffractometer curve presented by Resetarits et al[25]., indicates a doublet (strong peaks at 5.64 and 5.69Å).

The crystallographic parameters of estradiol hemihydrate are[37]:

$\underline{a}$ = 12.055 ± 0.003Å
$\underline{b}$ = 19.280 ± 0.003Å
$\underline{c}$ = 6.632 ± 0.002Å
$\underline{Z}$ = 4 $\underline{D}_x$ = 1.21g cm^{-3}
$\underline{D}_m$ = 1.20g cm^{-3}
orthorhombic, space group $\underline{P}2_12_12$.

The water molecules are located on the lattice binary axis, in association with the D-rings of steroidal molecules packed 'head-to-tail', and participate in the hydrogen bonding which supports the lattice. The differential thermal analysis (DTA) curve of estradiol hemihydrate exhibits endothermic peaks beginning at 112 and 174°C prior to the melting endotherm at 179°C (Fig. 6a)[42]; similar curves have been obtained using differential scanning calorimetry (DSC)[25,38,43]. Simultaneous effluent gas analysis of DSC specimens indicated that the two pre-melting endotherms were associated with solvent loss[38,43]. Thermogravimetric analysis was inconclusive (due to the small mass changes involved), but Karl Fischer titration of a sample which had been maintained for 1h at 148°C (i.e. above the temperature of the first DTA peak, which was abolished) indicated 1.8%^{w}/w residual moisture (cf. 3.5%^{w}/w initially). Thermomicroscopic examination of hemihydrated crystals had suggested that some structural rearrangement occurred prior to melting[38]; the pre-melting endotherm-exotherm doublet on DTA (Fig. 6b) and DSC[25,43] curves obtained at low heating rates would support this

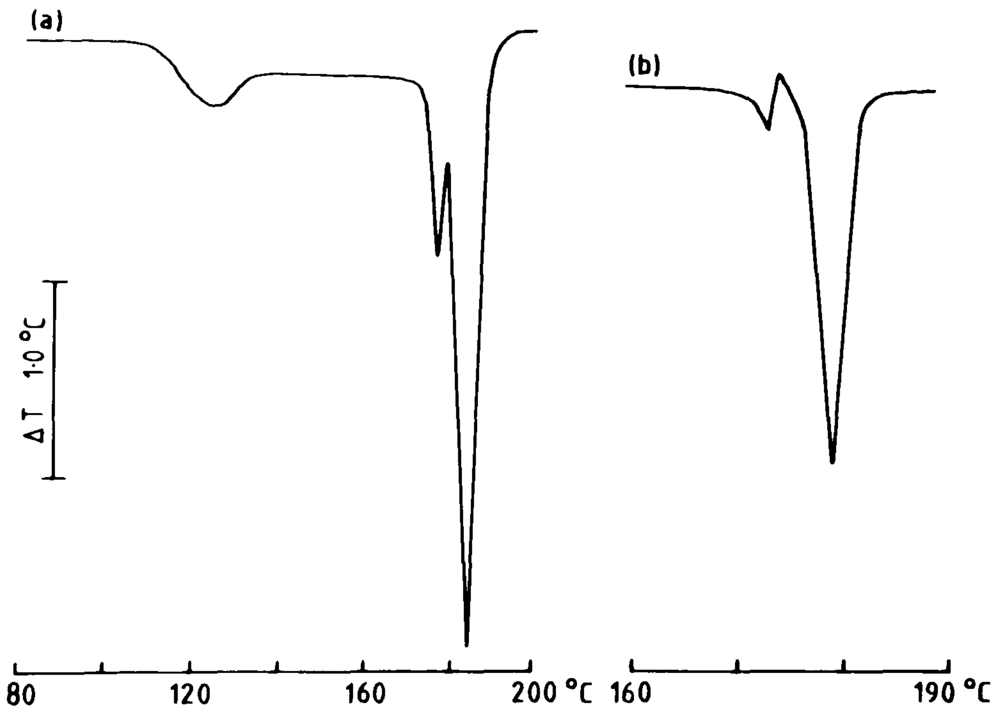
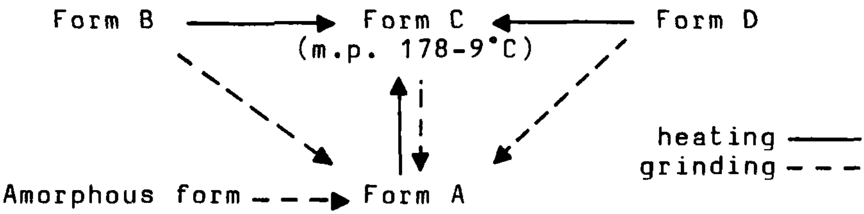


Fig. 6. DTA curve of estradiol hemihydrate at (a) 10° C. min.$^{-1}$ and (b) 2° C. min.$^{-1}$ heating rate: Stanton Redcroft 671B analyser, alumina reference, 8 mg. samples, open cups, static ambient atmosphere.

view. It appears then that estradiol hemihydrate desolvates in two stages, at about 112 and 174°C, the complete loss of lattice water resulting in simultaneous transformation to an anhydrous phase.

Smakula et al[10]., first reported the apparent polymorphism of estradiol; on the basis of X-ray powder diffraction and IR spectroscopy they identified four crystalline modifications (Forms A to D) and an amorphous, glassy phase, all of which transformed when subjected to heat and/or grinding, as summarised in the following scheme:



Independently, Kuhnert-Brandstatter[44] concluded (from the thermomicroscopic examination of melts, largely) that estradiol was dimorphic: Form II (m.p. 169°C) being the unstable monotrope of Form I (m.p. 178°C), the two modifications differing in habit, birefringence and IR spectrum[45]. Close scrutiny of the reports shows that Form A of Smakula et al., is in fact estradiol hemihydrate and Forms C and D correspond to Kuhnert-Brandstatter and colleagues' Forms I and II. It appears then that anhydrous estradiol is dimorphic and that in the process of desolvation the hemihydrate transforms to anhydrous Form I, the approximate interplanar-spaces and corresponding relative intensities (extrapolated from the X-ray powder diffractometer curve presented by Resetarits et al[25]., for a dehydrated sample) of which are:

d/Å	I/Io	d/Å	I/Io
7.63	3	4.23	3
6.44	5	4.06	3
5.77	10	4.01	3
5.55	4	3.88	1
5.26	3	3.71	1
4.86	4	3.32	2.
4.62	2		

As noted above, grinding transformed the anhydrous

modifications of estradiol to the hemihydrate, which is itself affected by comminution. Grinding crystalline estradiol hemihydrate resulted in no change in IR spectrum, but the DTA curve exhibited only a single, enlarged, pre-melting peak at about 120°C[42]; similar changes in DSC curves were reported for samples which had been milled (these also exhibited diffuse X-ray powder diffraction patterns)[43] or obtained by rapid precipitation from ethanol[25]. These changes suggest that comminution can structurally deform the crystalline hemihydrate to the extent that dehydration and simultaneous transformation to anhydrous Form I are facilitated, occurring at a temperature about 60°C lower than usual. (It has been noted that commercial 'micronized' estradiol apparently varies in degree of crystallinity, some batches, even from the same supplier, exhibiting 'deformed' DTA curves.)

Estradiol also forms solvates with organic solvents: a hemisolvate with methanol[38] and a monosolvate with ethanol[25], both of which desolvate (at 155 and 119°C, respectively) to Form I[38]. The crystallographic parameters of the monosolvate with propanol are[46]:

$\underline{a}$ = 12.215Å
$\underline{b}$ = 24.251Å
$\underline{c}$ = 6.671Å
$\underline{Z}$ = 4 space group $\underline{P}2_12_12_1$.

The presence of propanol in the lattice was found to only very slightly perturb the conformation of estradiol molecules[46], which may explain the apparent facility with which some organic solvents substitute for water of crystallisation.

Estradiol crystals precipitate in a variety of habits (Table 6); in view of its tendency to incorporate solvent of crystallisation, these very probably represent solvated forms, the hemihydrate in particular.

TABLE 6

Estradiol Crystal Habits[26,47]

Habit	Crystallisation solvent
Amorphous	Acetone, benzene, dioxane, ether.

Bladed	Dioxane, ethanol, aq.ethanol, ether, tetrahydrofuran.
Platy	Chloroform, dichloromethane, hexanol, isopropanol, methanol.
Prismatic	Benzene, chlorobenzene, methanol.

The outstanding solid-state characteristic of estradiol is the tenacity with which it adopts the hemihydrate form. In the experience of this author (and others[38,43]) commercial samples are invariably composed of this phase and, as described above, it has been demonstrated that water is selectively incorporated into the lattice when estradiol crystallises from solution, that anhydrous crystalline and amorphous phases transform to the hemihydrate (it was interesting to note that the DTA curve of shavings from the surface of a proprietary implant prepared by fusion was typical of the hemihydrate) and that dehydration is difficult, usually occurring completely only at temperatures close to melting. Clearly the presence of water molecules is a crucial requirement for the crystallographic stability of estradiol under normal conditions; on this basis it has been suggested that water may even play a significant part in the hormone's interactions with its receptors[48]. However, proper cognisance of this characteristic has not been taken by the major pharmaceutical compendia, although the NF XIV[49] stated that estradiol is 'hygroscopic' and the USP XXI[50] imposes a 3.5% limit on weight loss on drying at 105°C (at which temperature there may not be any discernable loss[38] or subsequent alteration in DTA curve). The evidence summarised here presents a substantial case for the molecular formula and relative molecular mass of estradiol to be properly acknowledged as :

$$C_{18}H_{24}O_2.{}^1/_2H_2O, \quad \underline{M}_r \; 281.39_5.$$

5. Methods of Analysis

5.1 Extraction and Purification

In biological fluids estradiol exists in the 'free'

form and conjugated as sulfates and glucuronides. Being an estrogen of relatively low polarity, estradiol can be quantitatively extracted with diethyl ether or benzene:petroleum ether, the organic phase washed with pH10.5 carbonate buffer to remove non-steroidal acidic impurities, then back-extracted into 0.1M sodium hydroxide and re-extracted into organic solvent after acidification with sulfuric acid. Its highly polar conjugates may be extracted with ethyl acetate (after separation of the unconjugated fraction) and hydrolysed by either refluxing with hydrochloric acid or incubation with sulfatases or β-glucuronidase enzymes[4].

Several techniques have been used to purify estradiol extracts, including precipitation as a complex with urea or digitonin (the latter is selective for the β-epimer; complexes may be cleaved with warm water and pyridine, respectively, and the steroid extracted), counter-current distribution and various chromatographic methods.

5.2 Absorption and Fluorescence Spectrophotometry

Several methods for the colorimetric analysis of estradiol have been developed on the basis of the Kober reaction, where a sample is heated with a phenol (e.g. β-naphthol) and sulfuric acid ($\geqslant$60%$^{w}/v$), the yellow solution diluted to an acid concentration of 30-50%$^{w}/v$ and re-heated to give a pink solution which absorbs maximally at around 530nm. In the more sensitive and accurate Ittrich modification, the re-heating after dilution is omitted and the coloured complex is extracted into 2%$^{w}/v$ p-nitrophenol in chloroform, giving a solution with yellow-green fluorescence. The natural fluorescence of estradiol is enhanced in concentrated sulfuric and other mineral acids[17]. Bartos and Pesez[51] comprehensively discuss the colorimetric and fluorimetric methods for estradiol.

5.3 Chromatography

The entire range of chromatographic techniques has been applied to the analysis of estradiol; paper chromatographic separations have been discussed at length by Bush[52] and Engel[17], and Stahl[53] lists a number of thin-layer systems, e.g.

adsorbent - silica gel G, 250μm layer,

		activated at 150°C for 3h;
solvent system	-	(a) chloroform:ethyl acetate, 80:20, (b) acetone: dichloromethane, 20:80;
location	-	100%w/w antimony trichloride in glacial acetic acid spray, developed at 95°C for 5min to bright red spots at (a) Rf0.32, (b) Rf0.56.

The development of modern chromatographic methods for estradiol continues apace. The powerful combination of gas or liquid chromatography and mass spectrometry is used to detect and assay estradiol in biological fluids[54], and several high-performance liquid chromatographic (HPLC) systems for the steroid and its metabolites have been developed[55,56].

5.4 Protein-binding Assays

A variety of these highly sensitive and (with care) specific methods have been developed for estradiol. Several radioimmunoassays[57] are available and developments in non-isotopic techniques[58], e.g. chemiluminescence immunoassays[59], have made them viable alternatives for routine analyses.

5.5 Bioassay

Initially, estradiol in biological fluids was assayed by biological methods based on changes in the vaginal epithelium or uterine mass of oophorectomized rodents[60]. Although often highly sensitive and specific (even in the presence of impurities), the precautions necessary for precision make them expensive and laborious and they are therefore now consigned to validating the physicochemical methods which have superseded. Modern cytochemical bioassays[61], though sensitive and elegant, have also not been adopted for routine use.

6. Stability

Estradiol can withstand boiling in dilute acids and alkali, and solid material remains chemically stable for at least five years under temperate conditions[62]. However, it has been reported that estradiol applied to silica gel TLC plates and exposed to the atmosphere for 1h underwent

substantial decomposition, but not primarily due to oxidation[63].

7. Metabolism, Pharmacokinetics and Bioavailability

Estradiol is biosynthesised in normal pre-menopausal women primarily by components of the ovaries (i.e. follicles, corpus luteum and stroma), following the classical route for steroids from acetate through cholesterol, pregnenolone and androstenedione (Fig. 7). Secretion varies with the phase of the menstrual cycle, is episodic and to a nycterohemeral rhythm, so plasma levels fluctuate rapidly; typical secretion rates during the follicular, midcycle and luteal phases are 80, 400 and 200μg d^{-1}, providing total estradiol plasma levels of about 6, 30 and 15ng dl^{-1}, respectively[64]. Only 1-3% of 'free' (i.e. unconjugated) hormone circulates unbound to protein, about 40% being bound to sex hormone binding globulin and 60% to albumin ($K=6.4\times10^{8}$ and 1.8×10^{5} M^{-1}, respectively)[65]. The extraglandular (principally in the liver, adipose tissue and skin) aromatisation of androgens is virtually the sole source of estradiol in post-menopausal women and accounts for about 70% of the steroid in males[64]. The production of estradiol by the fetoplacental unit during pregnancy has been reviewed[4].

A multiplicity of transformations are involved in the catabolism of estradiol, estrone (the subject of a recent Analytical Profile[66]) being a principal metabolite (Fig. 8). In humans, estradiol is excreted mainly in urine as glucuronide and sulfate conjugates[64], in which forms it also undergoes enterohepatic recirculation[67] so that although 23-68% of a dose may be recovered from bile as conjugates (mostly sulfoglucuronides), these are normally hydrolysed by mucosal enzymes and intestinal flora (mainly in the lower ileum and upper colon) and the free steroid mostly reabsorbed, leaving only 10-30% to be excreted in faeces (cf. 50-80% as conjugates in urine). Due to enterohepatic cycling the complete elimination of estradiol is delayed for 3-6d; the metabolic clearance rate in women is typically 1300l d^{-1} [64]. The decline in blood levels after intravenous infusion indicated that elimination was biphasic, with a half-life of 20 and 70min[68]; however the results after bolus injection showed that the curve

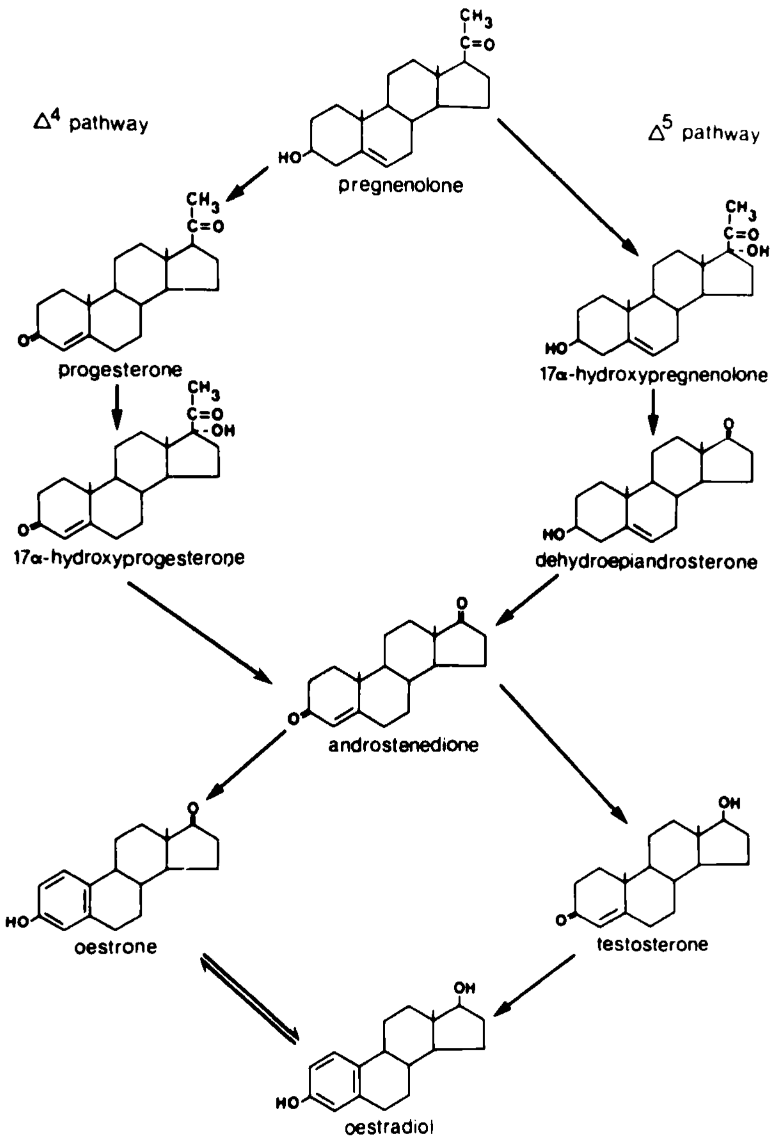


Fig. 7 Ovarian biosynthesis of estradiol[64].

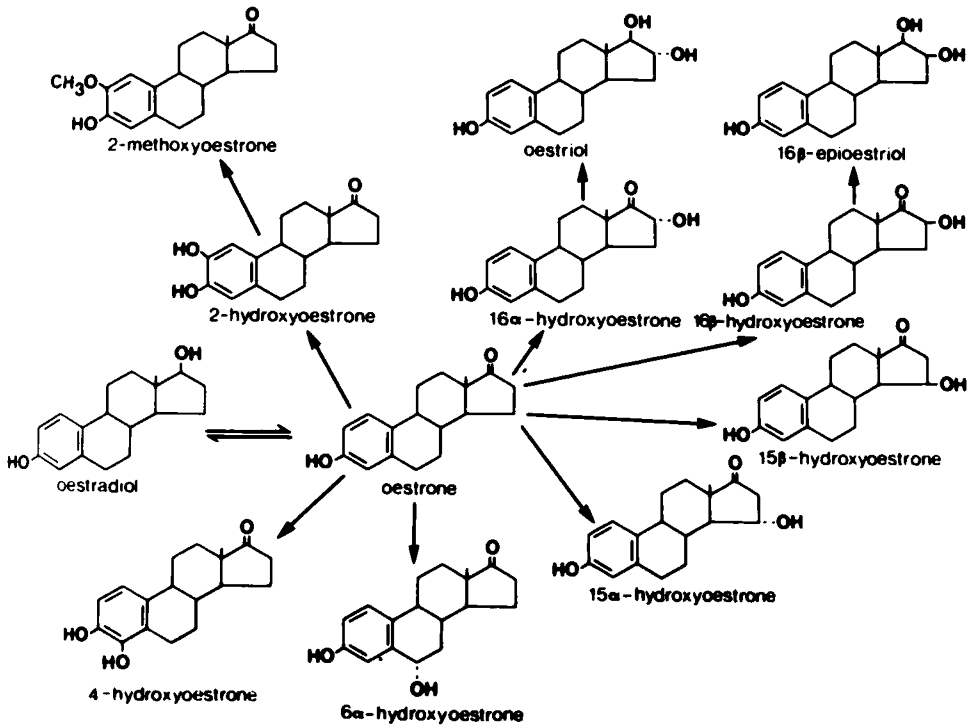


Fig. 8 Metabolism of estradiol[64].

was best described by the sum of three exponentials, the initial volume of distribution being 10.9(± 1.1 SEM)l[69].

To elicit a clinical response in replacement therapy (associated with plasma levels in the pre-menopausal follicular phase range, cf. 1ng dl^{-1} post-menopausally[64]), estradiol administered orally must be finely-divided, i.e. with an average particle size of only a few μm[70]. However, although capsules, aqueous suspensions and tablets of micronized material may be absorbed as rapidly as ethanolic solutions[71-73], the bioavailability of estradiol by the oral route is variable (with plasma concentrations peaking at 0.5-8h), incomplete (due to extensive intestinal and hepatic metabolism) and affected by diet and medication[74]. Oral administration also results in a substantial and sustained increase in circulating levels of estrone, an undesirable feature because of the apparent association with endometrial carcinoma[75]. Notwithstanding these disadvantages, the oral route remains clinically useful.

The intranasal instillation of an estradiol suspension in saline resulted in rapid absorption, but the rise in plasma levels was short-lived and accompanied by an increase in circulating estrone, suggesting local catabolism[76]. Similarly, absorption from tablets placed sublingually was rapid, a 0.5mg dose producing serum levels equivalent to 2mg orally, but again estrone levels were raised, perhaps due to catabolism within the reticuloendothelial system[77-79].

Estradiol is well absorbed from solid pellets prepared by fusion and implanted subcutaneously into the abdominal wall or buttocks, and has been administered in this form since the late 1930's[80,81]. Plasma levels can increase by 50% within an hour of implantation[82] and remain in the pre-menopausal range for several months[83,84] without large increases in circulating estrone occurring concomitantly, since first-pass metabolism is avoided. However, although estradiol implants are well tolerated (cf. progesterone, implants of which are expelled or form sterile abscesses in about 20% of patients) and can remain clinically effective for over three years[85], inter-patient variation in absorption is substantial[83] and they occasionally fail to produce a sustained response[82,84] (perhaps due to encapsulation in fibrous tissue[80,85], since

sometimes implants excised after several months appear in pristine condition[82]).

The vaginal route of administration has been advocated as the most appropriate for replacement therapy because, apart from beneficial local effects, systemic absorption occurs within minutes and, since the portal circulation is bypassed, levels of estrone are relatively low. However, despite early work on mice showing that estradiol was an order of magnitude more potent when administered intravaginally in aqueous glycerin than in olive oil[86], delivery by this route has yet to be optimised: nevertheless a variety of formulations have been used, including saline suspensions[76,87], creams[88], polysiloxane rings[89], hydrophilic pessaries[90] and even oral tablets[91].

Estradiol is readily absorbed through the skin; some parameters measured <u>in vitro</u> are[92,93] :

permeability constant -	$10^6 k_p$/cm h^{-1}
hydrated whole skin	3888
hydrated dermis	55080
hydrated stratum corneum	300
distribution ratio -	
stratum corneum/water	46.

Despite the mediocre partition coefficient, estradiol penetrates the stratum corneum within minutes but is only slowly, variably and incompletely absorbed into the systemic circulation after topical application[94,95]. This is because the percutaneous absorption of estradiol is highly dependent on the vehicle[27] (perhaps also the site and mode of application) and it is to some degree catabolised by the skin[96]. Nevertheless, although maximum plasma levels are achieved only after several hours, they are sustained (perhaps for days) due to the 'reservoir effect' of the stratum corneum and local retention in underlying dermal tissue, with the additional advantage of circulating estrone being maintained at low, pre-menopausal levels. Replacement therapy has been successfully achieved by the topical application of estradiol in hydro-alcoholic gels[94,95] and transdermal therapeutic systems[97,98].

8. <u>Determination in Body Fluids</u>

The entire analytical armamentarium is applied to

the analysis of estradiol in body fluids and tissues, and more specific methods are continually being developed. Colorimetric and fluorimetric methods based on the Kober reaction remain useful for routine assay of the relatively large amounts of estradiol in urine[99], but for blood and other fluids more specific and sensitive techniques are applied, notably radioimmunoassay, which has been applied to plasma[100], saliva[101] and faeces[102], and HPLC[55,56]. Although gas chromatography-mass spectrometry has been used to assay body fluids, e.g. semen[103], being the definitive method[54,104] it is usually reserved for special circumstances. The review by Bush[105] is recommended as an introduction to the complex and delicate business of estrogen extraction and analysis.

9. Determination in Pharmaceuticals

Physicochemical methods are usually chosen for the analysis of estradiol in medicines. Thin-layer chromatography remains useful for routine identification, as in stability testing of solid dosage forms[62]. Creams have been assayed by gas chromatography[106] and the highly fluorescent dansyl derivative of estradiol has been used to analyse solid and parenteral formulations directly[107] and after HPLC separation[108]. HPLC with a novel light-scattering detector may find application to pharmaceuticals[109].

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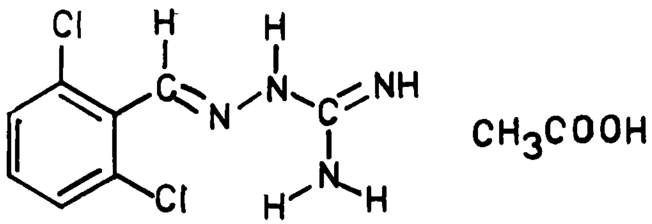
GUANABENZ ACETATE

Charles M. Shearer

1. Description

1.1 Name, Formula, Molecular Weight

The name used by Chemical Abstracts for guanabenz is hydrazinecarboximidamide, 2-[(2,6-dichlorophenyl)methylene]. It is also called (2,6-dichlorobenzylidene)aminoguanidine and N-(2,6-dichlorobenzylidene)-N'aminohydrazine (1). The drug code number (1) is NSC-68982 and the Chemical Abstracts Registry number is 5051-62-7 for guanabenz and 23256-50-0 for guanabenz acetate. This compound can exist (2) as the E-isomer (CAS-60329-04-6) or the Z-isomer (CAS:60329-05-7). Guanabenz acetate is the E-isomer.



$C_{10}H_{12}N_4O_2Cl_2$ Mol. Wt. = 291.14

1.2 Appearance, Color and Odor

Guanabenz acetate is a white to off-white practically odorless crystalline powder.

2. Physical Properties

2.1 Infrared Spectrum

An infrared absorption spectrum of a potassium bromide dispersion of guanabenz acetate (Wyeth Reference Lot C-12258) is presented as Figure 1. The spectral band assignments are listed in Table I.

Table I

IR Spectral Band Assignments

Wave number (cm^{-1})	Vibration Mode
3400	NH_2 stretch
3000 to 2600	protonated nitrogen stretch
1685	C=N stretch
1600	NH^+ and C=C stretch
1600 and 1395	COO^- stretch
780	three adjacent ring hydrogen deformations

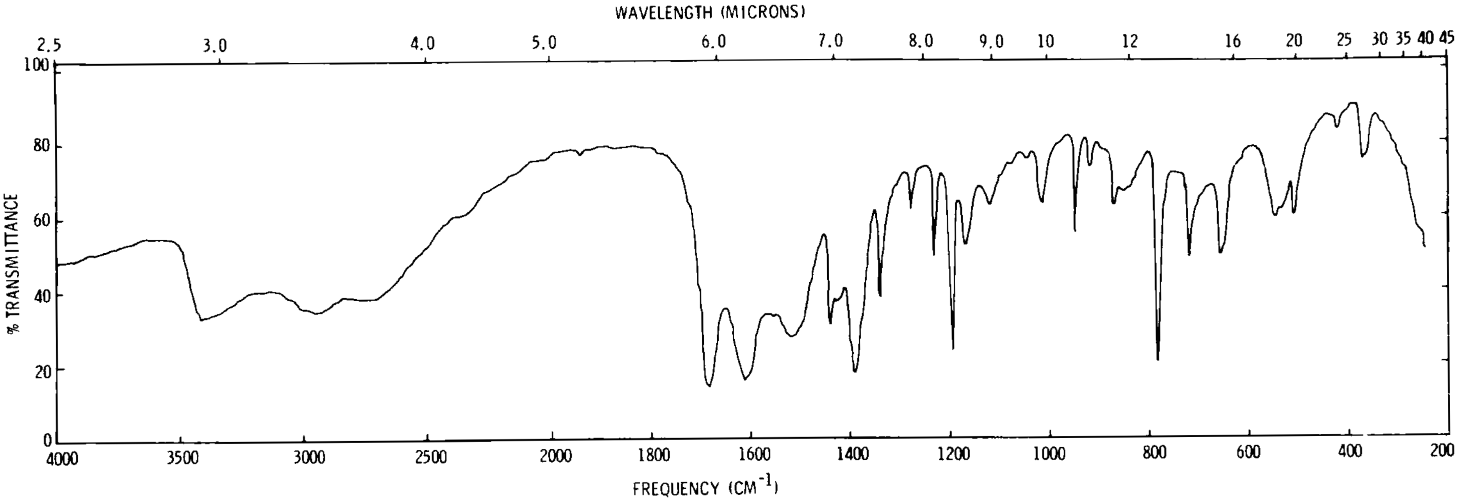


Figure 1 - Infrared Spectrum of Guanabenz Acetate,
(Wyeth Reference Standard, Lot C-12258) KBr pellet

The UV absorptivities found for guanabenz acetate (Wyeth Reference Standard, Lot C-12258) are given in Table III.

Table III

Ultraviolet Spectra Characteristics

Solvent	Max (nm)	Absorptivity
0.1N HCl	270	44.5
0.1N NaOH	295	43.4
absolute ethanol	312	45.2

2.4 Mass Spectrum

The mass spectrum of guanabenz acetate (Wyeth Reference Standard, Lot C-12258) was obtained (5) by direct injection of the sample into a MS-25 spectrometer. The ionizing beam energy was 70 eV. Figure 5 is a bar graph of the mass spectrum with the molecular ion at m/e 230. Identification of the pertinent masses is presented in Table IV.

Table IV

Mass Spectrum Fragmentation Pattern

m/e	Species
230	M+
195	M+ - Cl
153	M+ - Cl - CN_2H_2
123	M+ - Cl - CN_4H_4

2.5 Melting Range

Wyeth Reference Standard, Lot C-12258 of guanabenz acetate melts at 188 - 190°C (dec) by USP Class 1 (6). The Merck Index (1) reports a value of 192.5°C (dec).

2.2 Nuclear Magnetic Resonance Spectrum

The nuclear magnetic resonance spectrum sample (Wyeth Reference Standard, Lot C-12258) was prepared in deuterated dimethylsulfoxide containing tetramethylsilane as internal reference (3). The spectrum was obtained on a 300 MHz Varian XL-300 spectrometer and is presented as Figure 2. The spectral assignments are listed in Table II.

Table II

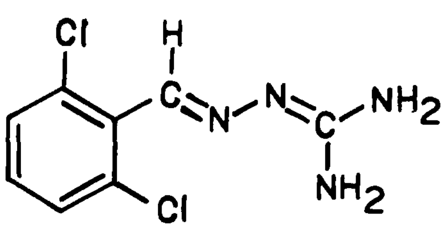
NMR Spectral Assignments

Assigned Proton	Chemical Shift (ppm)	Type	Number of Protons
O_2CCH_3	1.88	Singlet	3
Aromatic	7.25 to 7.60	Multiplet	3
Methine	8.26	Singlet	1
NH, OH exchangeable	6.00 to 9.5	Broad	5

2.3 Ultraviolet Spectra

The ultraviolet spectra of the neutral guanabenz species (in 0.1N NaOH) and the protonated species (in 0.1N HCl) are presented in Figure 3.

In nonaqueous solvents such as methanol or absolute ethanol (see Figure 4) guanabenz forms another tautomer and the spectrum is considerably changed (4). This tautomer is the more conjugated species with the following structure.



It was demonstrated that the ultraviolet spectra of an N-methylated guanabenz, which could not undergo such a tautomeric shift, had a maximum in either water or methanol near that shown by guanabenz in water.

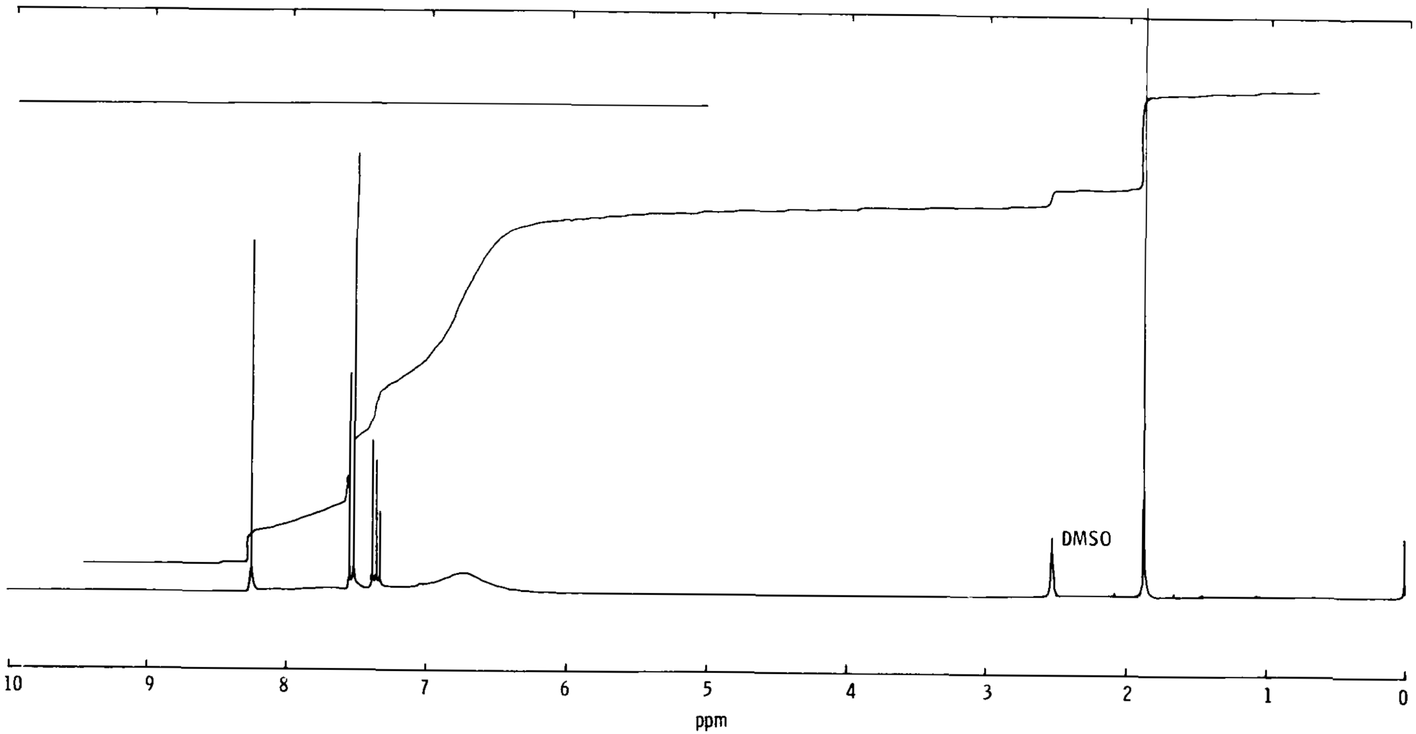


Figure 2 - NMR Spectrum of Guanabenz Acetate
(Wyeth Reference Standard, Lot C-12258) in
deuterated dimethylsulfoxide

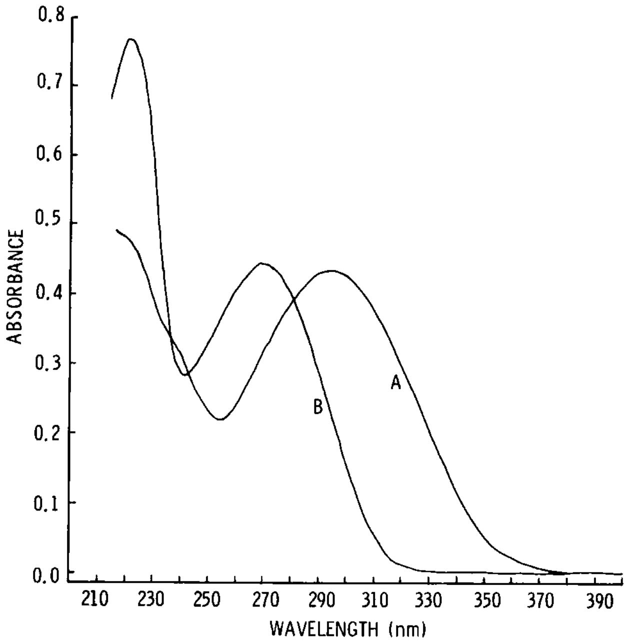


Figure 3 - Ultraviolet Spectra of Guanabenz Acetate (Wyeth Reference Standard, Lot C-12258) in (A) 0.1 N NaOH and (B) 0.1 N HCl

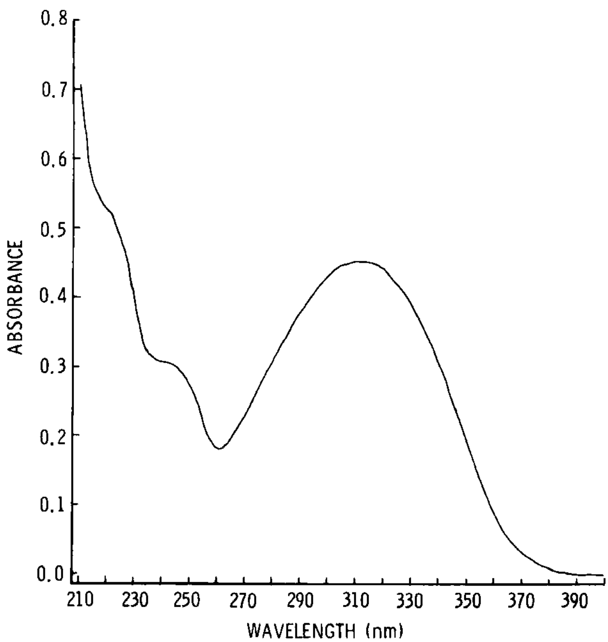


Figure 4 - Ultraviolet Spectrum of Guanabenz Acetate (Wyeth Reference Standard, Lot C-12258) in absolute ethanol

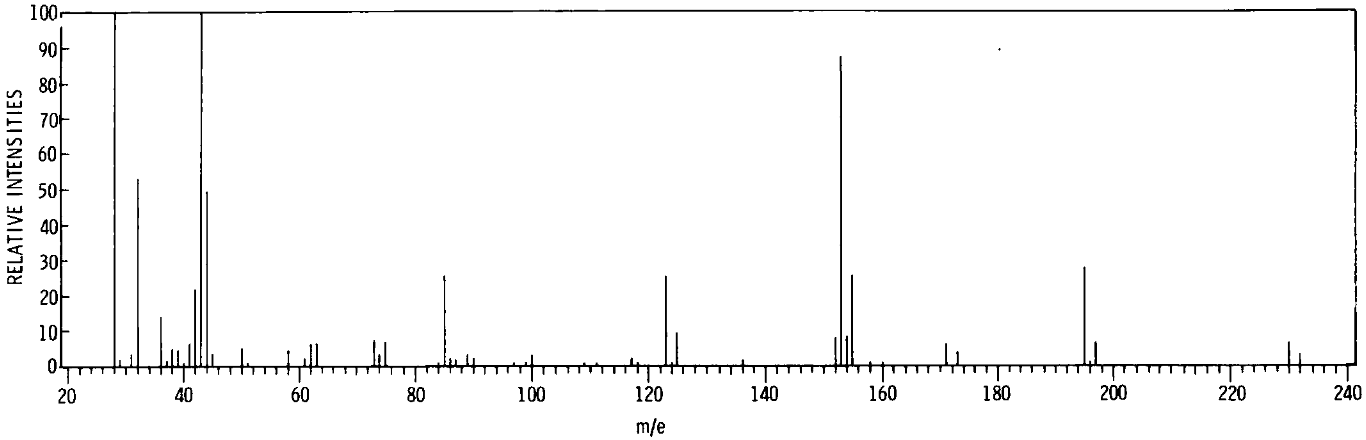


Figure 5 - Mass Spectrum of Guanabenz Acetate
(Wyeth Reference Standard, Lot C-12258)

2.6 Differential Scanning Calorimetry

The DSC thermogram of guanabenz acetate (Wyeth Reference Standard, Lot C-12258) is shown in Figure 6. The thermogram was obtained (7) at a heating rate of 10°C/min using a Perkin-Elmer DSC-2. The thermogram shows no endotherm or exotherm other than that associated with the decomposition melt.

2.7 Solubility

The following approximate solubilities at room temperature have been reported.

Solvent	Solubility (mg/mL)	Reference
Water	11	8
Alcohol	50	8
Propylene Glycol	100	8
Chloroform	0.6	9
Ethyl Acetate	1	9

2.8 Crystal Properties

The X-ray powder diffraction pattern of guanabenz acetate (Wyeth Reference Standard, Lot C-12258), obtained (7) with a Phillips diffractometer using CuKα radiation is presented as Figure 7. The calculated "d" spacings are given in Table V.

Table V

X-Ray Powder Diffraction Pattern

d	I/I_o	d	I/I_o
15.0	9	41.8	8
18.5	38	42.7	26
25.6	2	43.3	5
27.7	2	45.3	15
29.1	4	46.9	57
31.0	11	47.7	14
31.3	14	51.2	10
33.1	100	53.5	7
34.2	30	55.7	3
35.8	90	56.3	3
40.2	85	58.3	10
		58.6	10

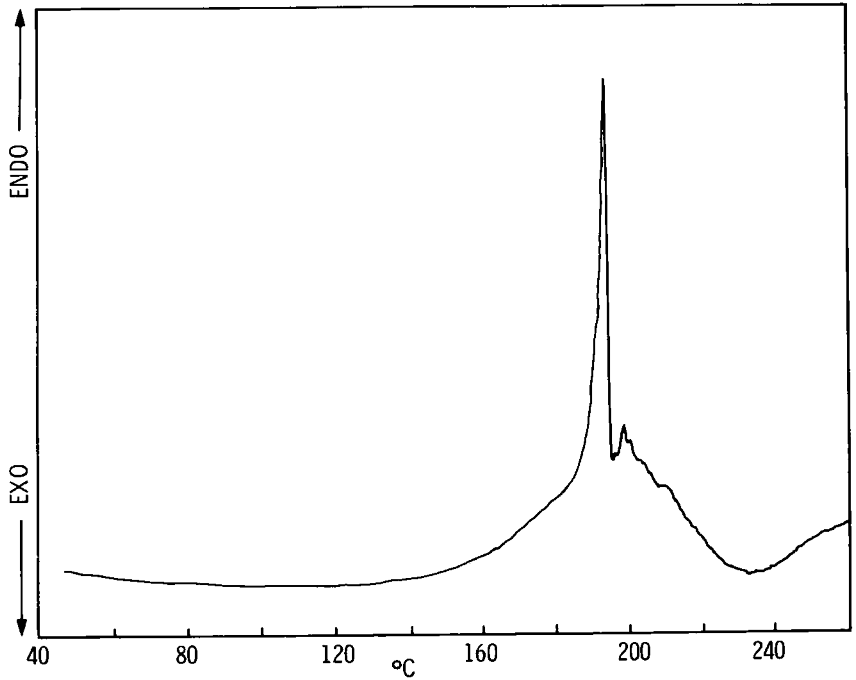


Figure 6 - Differential Scanning Calorimetric Thermogram of Guanabenz Acetate (Wyeth Reference Standard, Lot C-12258)

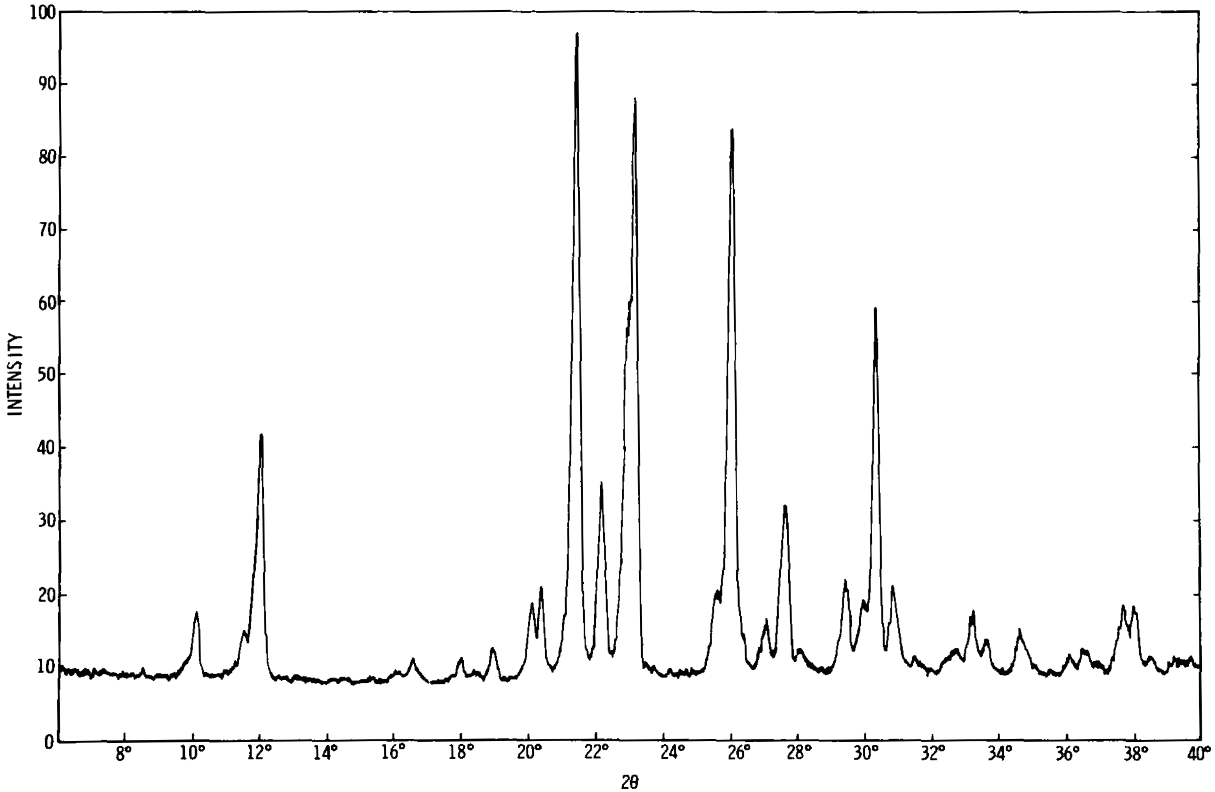
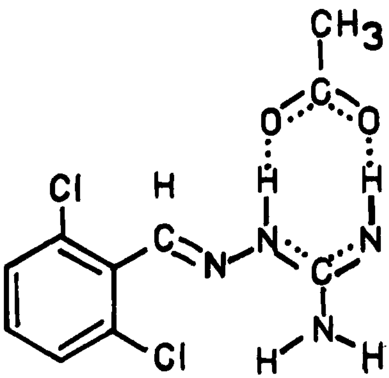


Figure 7 - X-Ray Diffraction Pattern of Guanabenz Acetate
(Wyeth Reference Standard, Lot C-12258)

The single crystal X-ray crystallographic structure determination of guanabenz acetate indicates that guanabenz (base) and acetic acid are hydrogen bonded and that the entire salt lies substantially within a single plane (10). On the basis of bond lengths and angles the bonding for guanabenz acetate in the crystal may be represented as given below.



The crystal system is orthorhombic, space group of $^{2}Pbca\text{-}D^{15}_{2R}$ (No. 61). The lattice constants are A= 17.326(3) Å, b=13.436(2) Å and c=10.999(2) Å with eight molecules per unit cell.

2.9 Dissociation Constants

The ionization constants for guanabenz acetate were determined by potentiometric titration [method of Parke and Davis (11)] in 40% ethanol/water to be 5.8 (acetate) and 8.1 (guanidinium). Spectrophotometric determination in water for the guanidinium ion also gave 8.1 (12).

2.10 Electrochemical Properties

Guanabenz acetate at a concentration of 0.1 mg/mL is reduced with a half-wave potential of about -0.9V vs SCE in 0.1M acetic acid and about -1.1V vs SCE in 0.1M NaH_2PO_4. The half-wave potentials and the limiting diffusion currents are concentration dependent in these solvents (12).

3. Synthesis

Guanabenz can be synthesized (13) as the acetate by the condensation reaction of 2,6-dichlorobenzaldehyde with aminoguanidine in the presence of acetic acid.

4. Stability and Degradation

Guanabenz acetate can decompose by hydrolysis to the starting materials in its synthesis, aminoguanidine and 2,6-dichlorobenzaldehyde. It can also hydrolyze forming 2,6-dichlorobenzaldehyde semicarbazone. These reactions occur very slowly even under reflux in strong acid or base (2).

It has been shown by spectral evidence that guanabenz acetate exists in the more stable E-configuration (2). The Z-isomer is formed from guanabenz by irradiation in solution with ultraviolet light (2,14). The reaction is reversible in the presence of acid (14) and an equilibrium between the two isomers is formed (2).

5. Metabolism and Pharmacokinetics

5.1 Metabolism

In man the major metabolite of guanabenz is (E)-p-hydroxyguanabenz and its glucuronide. Other identified metabolites are Z-guanabenz, (Z)-p-hydroxyguanabenz, 2,6-dichlorobenzyl alcohol glucuronide and guanabenz glucuronide. These metabolites and those reported below for studies in the Rhesus monkey were characterized by comparison of their Rf's in thin-layer chromatography to that of authentic sample (15).

The following metabolites have been identified (16) following administration of guanabenz to Rhesus monkeys: Z-guanabenz, 2,6-dichlorobenzyl alcohol, 2,6-dichlorobenzaldehyde, p-hydroxyguanabenz and its glucuronide conjugate and 2,6-dichlorobenzaldehyde azine.

After oral or i.v. administration of guanabenz acetate to rats, thin-layer chromatographic analysis of urine and bile revealed the metabolites: Z-guanabenz, 2,6-dichlorobenzyl alcohol, p-hydroxyguanabenz, 3-hydroxyguanabenz, and sulfates and/or glucuronides of these (17).

5.2 Pharmacokinetics

Meacham et al. (15) reported on the disposition of ^{14}C guanabenz in hypertensive patients. Maximum concentrations of guanabenz were reached at 2 to 5 hours after dosing. The major route of elimination was into urine (80%), but guanabenz itself accounted for less than 1% of the total. Kinetic parameters for guanabenz were estimated by fitting the plasma and urine data to a 2-compartment model.

A study similar to the one described above, but using Rhesus monkeys was also described by Meacham et al. (18). The results of this study, which were similar to those obtained in human patients, indicates that the Rhesus monkey may serve as a satisfactory model for man in disposition studies of guanabenz.

The pharmacokinetics of guanabenz in rats were reported by De Marchi (19) and by Yokozama (20,21).

6. Identity

Infrared spectroscopy can be used directly upon the drug substance for its identification.

Thin-layer chromatography, ultraviolet and nuclear magnetic resonance spectrophotometry will readily distinguish guanabenz from the Z-isomer (2).

7. Methods of Analysis

7.1 Elemental Analysis

The elemental analysis of guanabenz acetate (Wyeth Reference Standard, Lot C-12258) is presented below.

Element	% Calculated	% Reported (22)
C	41.26	41.00
H	4.15	4.05
N	19.24	19.34
Cl	24.35	24.50

7.2 Phase Solubility Analysis

Phase solubility analysis (23) on guanabenz acetate (Wyeth Reference Standard, Lot C-12258) using 12% methanol in acetone as the solvent gave a purity of 100 $\pm$ 0.4%.

7.3 Direct Spectrophotometric Analysis

A stability-indicating assay by ultraviolet spectrophotometry has been reported (2). It will assay intact guanabenz in the presence of its degradation products.

7.4 Titrimetric Analysis

Guanabenz acetate can be titrated with perchloric acid in acetic acid after dissolving the sample in glacial acetic acid (24,25).

7.5 Chromatographic Analysis

7.51 Thin-Layer Chromatography

The following systems using silica gel plates have been reported:

Solvent	R_f	Reference
chloroform/methanol (10:1)	0.7	14
chloroform/methanol/ ammonium hydroxide (48.5:50:1.5)	0.71	18
ethyl acetate/methanol/ water/ammonium hydroxide (68:26:4:2)		15
benzene/acetone/acetic acid (70:10:20)		15
chloroform/methanol/ ammonium hydroxide (60:40:1)		25

7.52 Gas Chromatography

Knowles et al. (26) describe a sensitive gas chromatographic assay for guanabenz in urine and plasma. The method involves the acid hydrolysis of guanabenz to 2,6-dichlorobenzaldehyde which is chromatographed on a 2 mm i.d. x 3 m column packed with 1% neopentylglycol succinate on 60/80 mesh Chromosorb G AW-DMCS at 200° and detected by an electron capture detector.

7.53 High Performance Liquid Chromatography

Guanabenz can be separated (2,27) from its degradation products by an eluant consisting of 430 mL methanol/570 mL distilled water/3 mL phosphoric acid on a octadecyl reverse-phase column.

7.6 Radioligand Binding Analysis

Fluck et al. (28) describe a radioligand assay specifically for guanabenz. It is based upon its differential binding of guanabenz and its metabolite to cerebral α_2 receptors. It can be used in analysis of guanabenz in plasma and urine. It will distinguish guanabenz from the Z-isomer.

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IODAMIDE

Davide Pitré

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

Benzoic acid, 3-(acetylamino)-5-[(acetylamino) methyl] -2,4,6-triiodo -
n-Toluic acid,α, 5-diacetamido-2,4,6-triiodo
CAS Reg. N. [440-584]

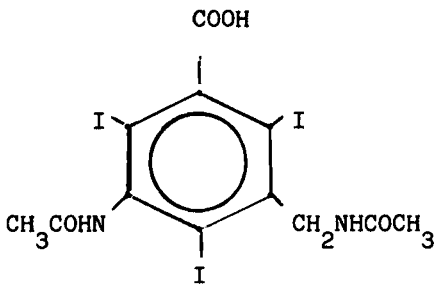
1.1.2 Generic Names

Iodamide (BAN, DCF, USAN)
Iodamidum (NFN)
Ametriadinic acid
Deriv.
Iodamide Meglumine (USAN)

1.1.3 Trade Names

Angiomiron (Schering AG-Berlin)
Contraxin (Takeda - Osaka)
Isteropac (Bracco-Milano)
Opacist (Bracco-Milano)
Uromiro (Bracco-Milano)
Uromiron (Schering A.G.-Berlin)
Urombrine (Dagra-Dienmarc
- Trade names of 1-deoxy-1-(methylamino)-D-glucitol salt
    Renovue (Squibb)
    Jodomiron (Erco-Vedback)

1.2 Formula, Molecular weight



$C_{12}H_{11}I_3N_2O_4$ Mol.wt. 627.93

1.3 Appearance, Color, odor

White, fine, crystalline powder, odorless and with a very bitter taste (2).

2. Physical Properties

2.1 Spectra

2.1.1 Infrared Spectrum

The infrared spectrum in potassium bromide pellet was reported by E. Felder et al (3). The spectral band assignments are reported in Table I.

Table n. 1

Values of infrared absorption

Frequency (cm^{-1})	Type of vibration	Assignment
3380 3200	ν_{NH}	-CONH groups
2980÷2500	ν_{CH} and ν_{OH} ass	$-CH_2$, $-CH_3$ and -COOH groups
1728	$\nu_{C=O}$	-COOH group
1660 1635	$\nu_{C=O}$	-CONH "Amide I band" groups
1525	$\delta_{NH} + \nu_{CN}$	-CONH "Amide II band"groups
1425	$\delta_{OH} + \nu_{C-O}$	-COOH group
1460	δ_{CH}	$-CH_2$ group
1370	δ_{CH}	$-CH_3$ group
1296 1280	$\nu_{CN} + \delta_{NH}$	-CONH "Amide III band"groups
1210 1193	$\delta_{OH} + \nu_{C-O}$	-COOH group
985		
758		
738		
695		
685		
650		

2.1.2 Nuclear Magnetic Resonance Spectra

2.1.2.1 ^{1}H-NMR

The ^{1}H-NMR spectrum of Iodamide, recorded in DMSO solution with a EM 390 Varian spectrometer at 90 MHz, is reported in Fig.1 (4). The spectral assignment is shown in Table 4.

Table N. 2
^{1}H-NMR data

Chemical shift δ_H (ppm, TMS)	Multiplicity	n. Protons	Assignment
10.00	s	1, exch.	ø-NHCO-
7.93	t	1, exch.	ø-CH_2<u>N</u>HCO
4.67	d	2	ø-CH_2
3.0÷4.0	v.l.	~1	-COOH
2.01	s	3	ø-NHCO<u>CH</u>$_3$
0.90	s	3	ø-CH_2-NHCO<u>CH</u>$_3$

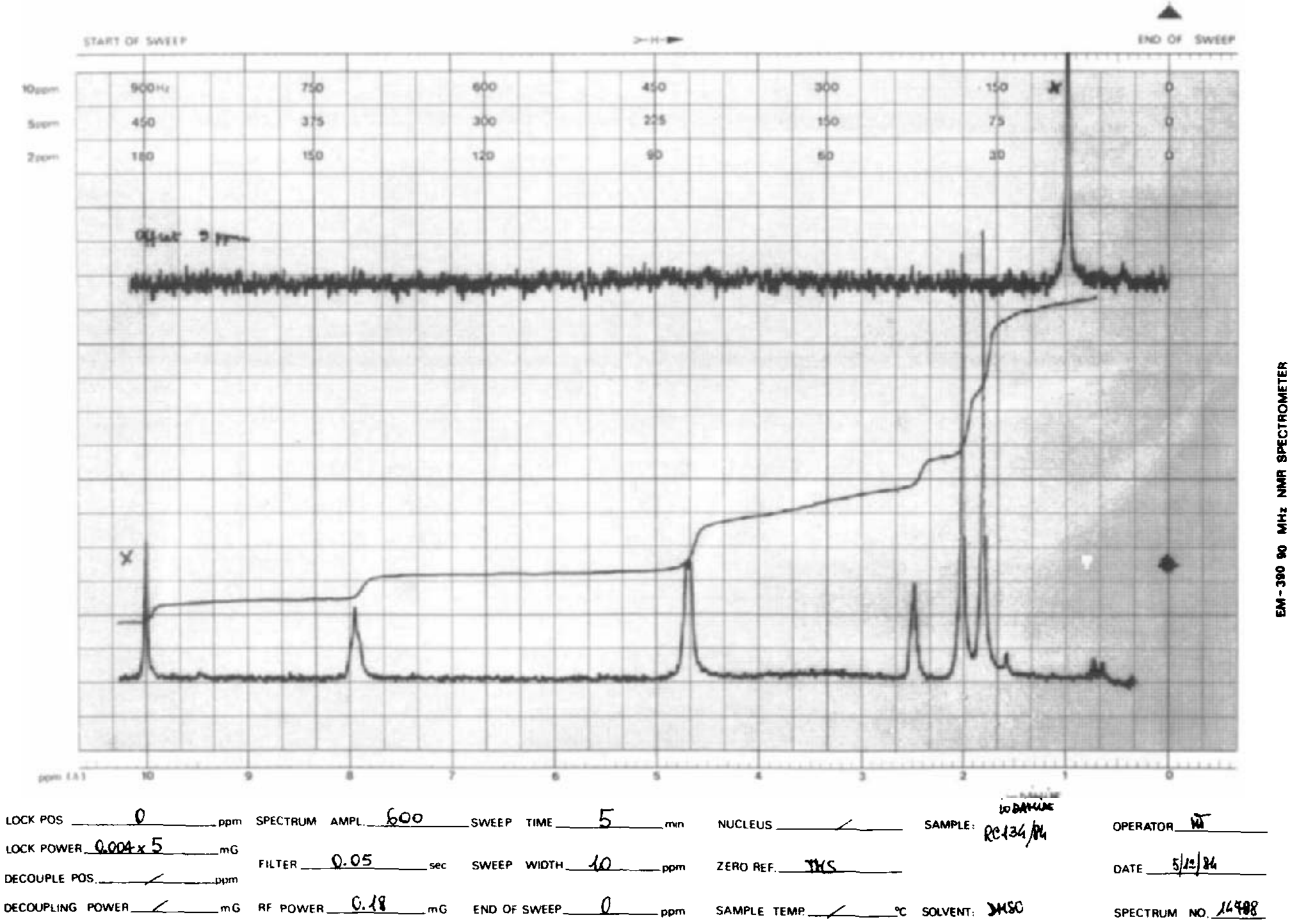


Fig. 1 - ^{1}H-NMR Spectrum of Iodamide in DMSO - d_6

2.1.2.2 ^{13}C-NMR

The ^{13}C-NMR spectrum, recorded in DMSO solution with a XL-100 Varian spectrometer at 25.2 MHz, is shown in Fig.2. The assignments are presented in Table 3 (4).

Table n. 3
^{13}C-NMR data

Line n.	Intensity	Chemical schift δC(ppm, TMS)	Assignment
1	84	169.6	-COOH
2	100	168.8	
			ØNH$\underline{CO}$ + ØCH$_2$NHCO
3	86	167.5	
4	27	149.0	C_1-C_3 and C_5 aromatic
5	77	143.7	
6	104	143.6	carbon
7	39	107.5	C_2-C_4 and C_6 aromatic
8	35	97.0	
9	47	95.8	carbon
10	32	56.1	Ø-$\underline{CH_2}$
11	69	22.8	Ø-CH2NHCO$\underline{CH_3}$
12	74	22.0	Ø-NHCO$\underline{CH_3}$

2.1.3 Mass Spectrum

The main fragmentation patterns observed in EI/MS were reported by E. Felder et al (3). In order to better characterize the molecular ion which in the EI spectrum is very scarcely abundant (0.001%), the mass spectrum of iodamide was recorded using a Fast Atom Bombardment (VG Micromass 70-70E, thioglycerol mixture). The normalized spectrum is shown in Fig. 3 from which it appears that the pseudomolecular ion $[M + H]^+$ at m/z = 629 represents the base peak.

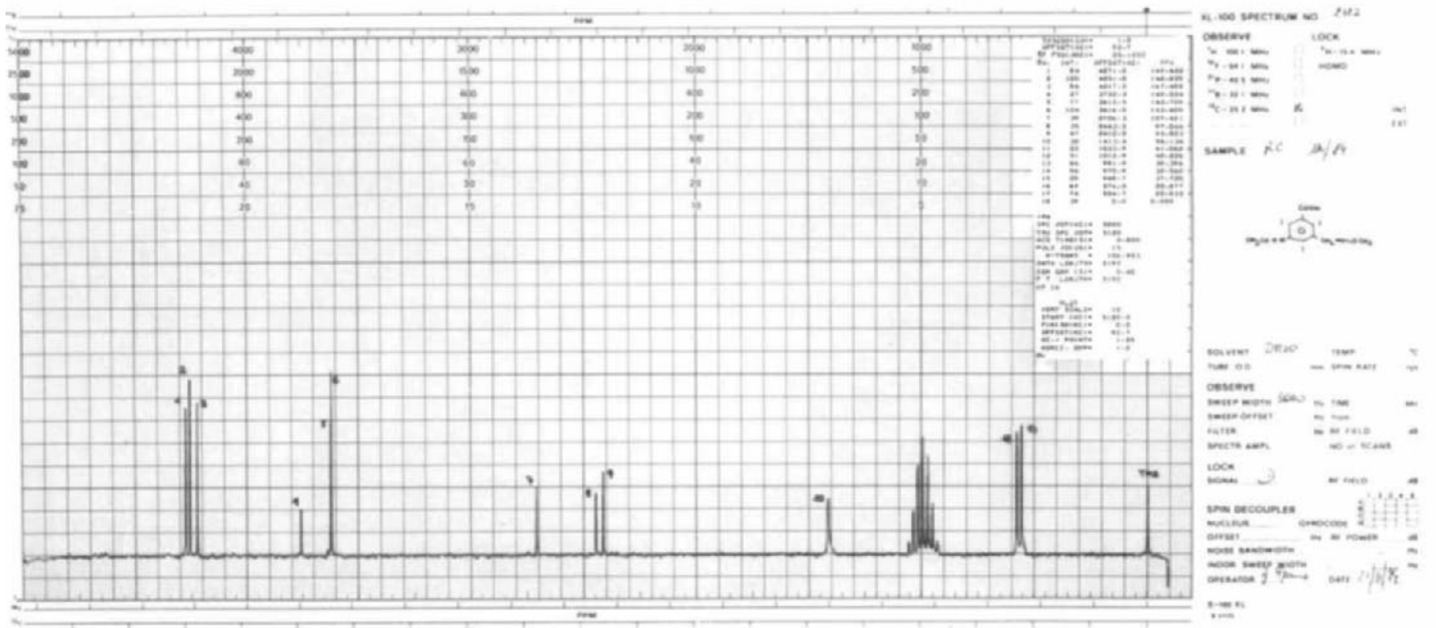


Fig. 2 - ^{13}C-NMR Spectrum of Iodamide in DMSO - d_6

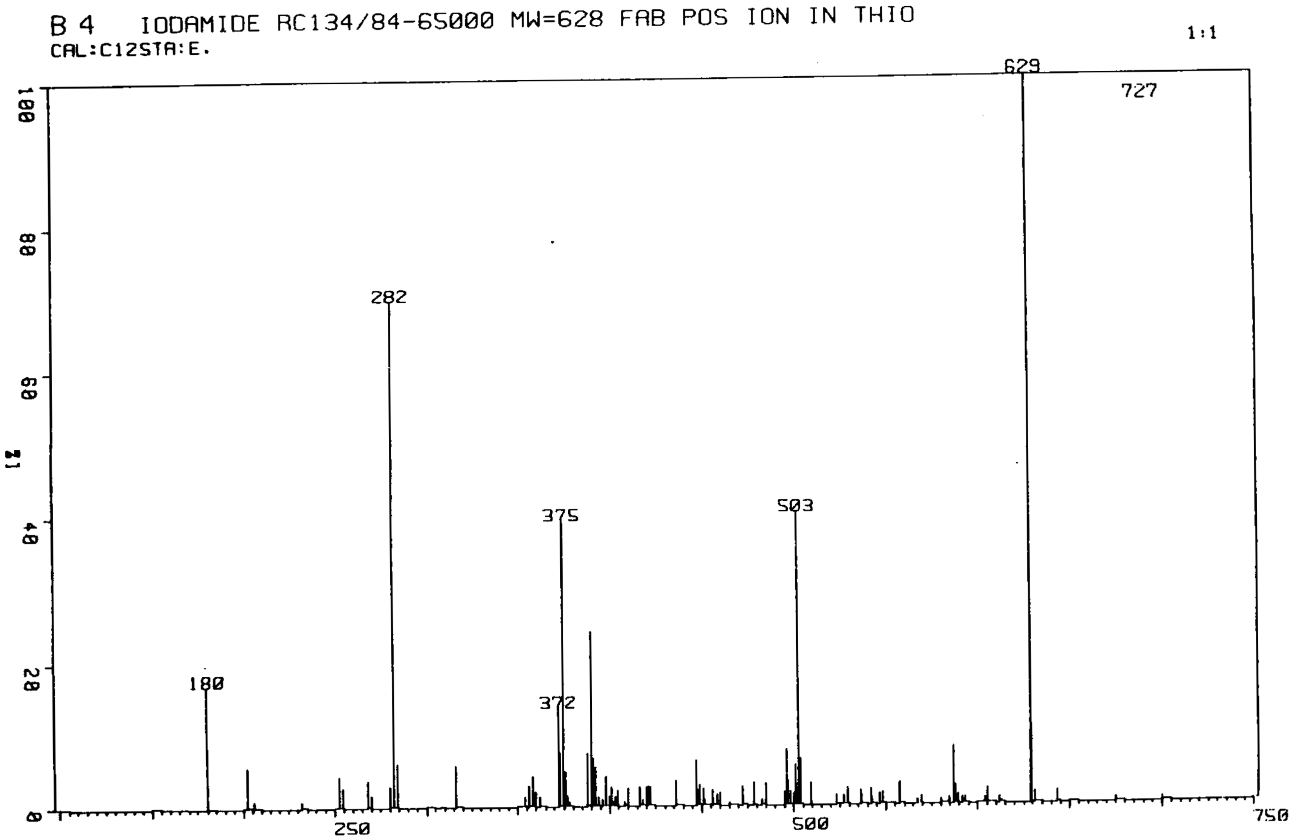


Fig. 3 - Positive FAB/MS spectrum of Iodamide

2.1.4 Ultraviolet Spectrum

The ultraviolet absorption spectra of Iodamide in methanol, 0.1N NaOH and 0.1N HCl were reported by E. Felder et al (3). The relevant spectrophotometric data are reported in Table 4.

Table n. 4

Solvent	λ max (nm)	lg ε max	$E_{1\,cm}^{1\%}$ (λmax) found	s	n
Methanol	238.0	4,520	527.3	3.60	20
0.1 N NaOH	236.1	4,572	593.9	2.40	12
0.1 N HCl	237.6	4,502	505.4	0.88	5

2.2 Physical Properties of Solid State

2.2.1 Differential Thermal Analysis

Differential thermal analysis was carried out in a Perkin-Elmer model DSC-1 calorimeter(heating rate of 4°/min). In the temperature range from 40°-350° only one endothermic transition was seen at 284°. A thermogravimetric analysis carried out under nitrogen with RH Cahn electrobalance and a Stanton-Redcroft temperature programmer showed decomposition with loss of iodine at 280° (3).

2.2.2 X-Ray Powder Diffraction

The X-ray powder diffraction pattern of Iodamide was determined with a Philips Powder Diffractometer PW 1710 using nichel-filtered copper radiation with the following instrumental conditions: tube LFF, Cu, 40 KV, 40 mA; slits 1°-0.1 mm-1°; detector PW_3 1171 proportional counter plus discriminator; scale $2x10^3$ cps; time constant 1"; scanning speed 0.004°/sec; paper speed 1 cm/1° 2θ; specimen holder Niskanen plus internal standard.

Interplanar distances (d(Å)=1.54051/2d Sin ϑ) and relative intensities are reported in Table 5.

Table n. 5
X-Ray Powder Diffraction Pattern of Iodamide

N	d(Å)	I/I_ox100	N	d (Å)	I/I_ox100
1	10.52	70	31	2.92	11
2	8.91	2	32	2.91	10
3	7.11	6	33	2.86	12
4	6.32	5	34	2.81	6
5	6.04	4	35	2.75	3
6	5.75	2	36	2.69	4
7	5.70	3	37	2.65	2
8	5.43	2	38	2.63	11
9	5.26	100	39	2.59	4
10	5.14	8	40	2.56	4
11	5.02	12	41	2.54	10
12	4.64	6	42	2.48	3
13	4.21	11	43	2.43	3
14	4.14	2	44	2.40	3
15	4.07	3	45	2.38	3
16	4.03	4	46	2.35	3
17	3.86	14	47	2.31	4
18	3.81	6	48	2.29	3
19	3.77	4	49	2.28	3
20	3.65	5	50	2.20	3
21	3.50	12	51	2.13	3
22	3.43	6	52	2.11	7
22	3.43	6	53	2.01	5
23	3.31	8	54	1.96	4
24	3.22	3	55	1.93	3
25	3.19	9	56	1.75	5
26	3.12	4	57	1.72	2
27	3.07	4	58	1.71	2
28	3.03	4	59	1.67	2
29	2.98	2	60	1.61	2
30	2.96	3	PLUS OTHER LINES		:2

2.2.3 Melting Range

Iodamide shows in thermal microscopy a melting range between 200° and 270°, with decomposition (3). M.p. 255-7° (5).

2.2.4 Eutectic Temperature

The following melting points of Iodamide, that have the advantage of being very sharp, have been reported (3)

Mixture	M.p.
N-Phenylacetamide	110.1°
p-Ethoxyacetamide	131.8°
Salophen	186.7°
1-Cyanoguanidine	183.0°
N-Phenylbenzamide	158.8°

2.3 Solution Data

2.3.1 Solubility

Iodamide is sparingly soluble in methanol, slightly soluble in water and in ethanol and practically insoluble in ether and in chloroform (2).
Water solubilities, expressed in g/100 ml, are 0.30 at 20°, 0.32 at 40° and 0.39 at 60°.
Water solubilities, as a function of pH, are reported in Table n. 6 (3).

Table n. 6
Solubility in water as a function of pH at 20°

pH ($\pm$0.05)	Solubility g/100 ml
1.0	0.03
2.0	0.14
3.0	1.16
3.6	5.41
3.8	11.1
4.0	18.7
4.5	52.9
5.0	73.2

2.3.2 pKa

The apparent ionization constant, determined in methylcellosolve/water (W/W) was $pK^{25}_{MCS} = 4.15$; extrapolation to aqeuous solution gave a $pK^{25}_{H_2O} = 1.88$ (7).
Other Authors (6) reported a pKa = 3.7, determined by titration.

2.3.3 Partition Coefficients

The n-octanol/buffer partition coefficients at pH between 1 and 8 are listed in Table 7 (3).

Table n. 7
n-octanol/aqueous phase partition coefficients

Aqueous phase	Part. Coeff.
0.1 M Citrate buffer pH 1	2.71
0.1 M Citrate buffer pH 2	1.02
0.1 M Citrate buffer pH 3	0.12
0.1 M Citrate buffer pH 4	0.02
0.1 M Citrate buffer pH 5	≤0.01
0.1 M Phosphate buffer pH 6	≤0.01
0.1 M Phosphate buffer pH 7	≤0.01
0.1 M Phosphate buffer pH 8	≤0.01

A partition coefficient of 0.9×10^{-3} in chloroform/water at pH 3.3 was reported (6).

2.3.4 Index of Refraction

The refraction index (n_D^{20}) of the aqueous solutions of the meglumine and sodium salts of Iodamide is given in Table 8.

2.3.5 Specific Gravity

The specific gravity (d_{20}^{20}) of water solutions of the meglumine and sodium salts of Iodamide is reported in Table 8.

2.3.6 Osmotic Properties

Osmolalities of aqueous solutions of the sodium and meglumine salts of Iodamide are reported in Table 8 (4).

Table n. 8
Osmotic properties
Sodium salt of Iodamide (M.W. = 650.0)

Iodamide-Na g/100 ml	mg I/ml	d^{20}_{20}	n^{20}_{D}	Osmol/kg
5.00	29.3	1.0282	1.3410	0.1464
10.00	58.6	1.0595	1.3493	0.2869
20.00	117.2	1.1217	1.3658	0.5668
30.01	175.8	1.1850	1.3822	0.8740
40.00	234.3	1.2467	1.3989	1.202
50.01	293.0	1.3092	1.4152	1.591
60.00	351.5	1.3707	1.4314	2.025
70.01	410.1	1.4347	1.4488	2.527
80.00	468.6	1.4979	1.4645	3.092

Meglumine salt of Iodamide (M.W. = 823.2)

Iodamide-MGA g/100 ml	mg I/ml	d^{20}_{20}	n^{20}_{D}	Osmol/kg
10.09	46.6	1.0499	1.3491	0.2234
20.17	93.1	1.1052	1.3655	0.4346
30.24	139.6	1.1593	1.3819	0.6532
40.33	186.2	1.2133	1.3989	0.8955
50.42	232.8	1.2671	1.4158	1.165
60.48	279.2	1.3202	1.4325	1.490
70.57	325.8	1.3749	1.4490	1.884
80.63	372.3	1.4297	1.4652	2.370

3. Manufacturing Procedures

3.1 Synthesis

The starting step in the synthesis of Iodamide (1) (1) is the introduction of an acylaminomethyl group in position 5 of a benzoic acid molecule, either substituted or not in position 2 and/or 4 with a chlorine atom, followed by the introduction of an amino group in position 3 by means of nitration and reduction.
The compound thus obtained is subsequently iodinated and acetylated. If the starting product is benzoic acid (x = H, X_1 = H) the synthesis has no industrial value because the reaction leads mostly to an undesired compound with the nitro group in position 4. The synthesis was therefore studied using 2-chloro benzoic acid (X = Cl, X_1 = H), 4 chlorobenzoic acid (X = H, X_1 = Cl) and 2,4-dichlorobenzoic acid (X = X_1 = Cl) as a starting products in which the introduction of the chain is easier. Nowdays the industrial synthesis is performed starting from 2-chlorobenzoic acid using the scheme through 3-acetylaminomethyl-6-chlorobenzoic acid (IVb). This product is nitrated to (Vb), reduced to (VI) and iodinated to 3-acetylaminomethyl-5-amino-2,4,6-triiodobenzoic acid (VII) which is finally acetylated to Iodamide.

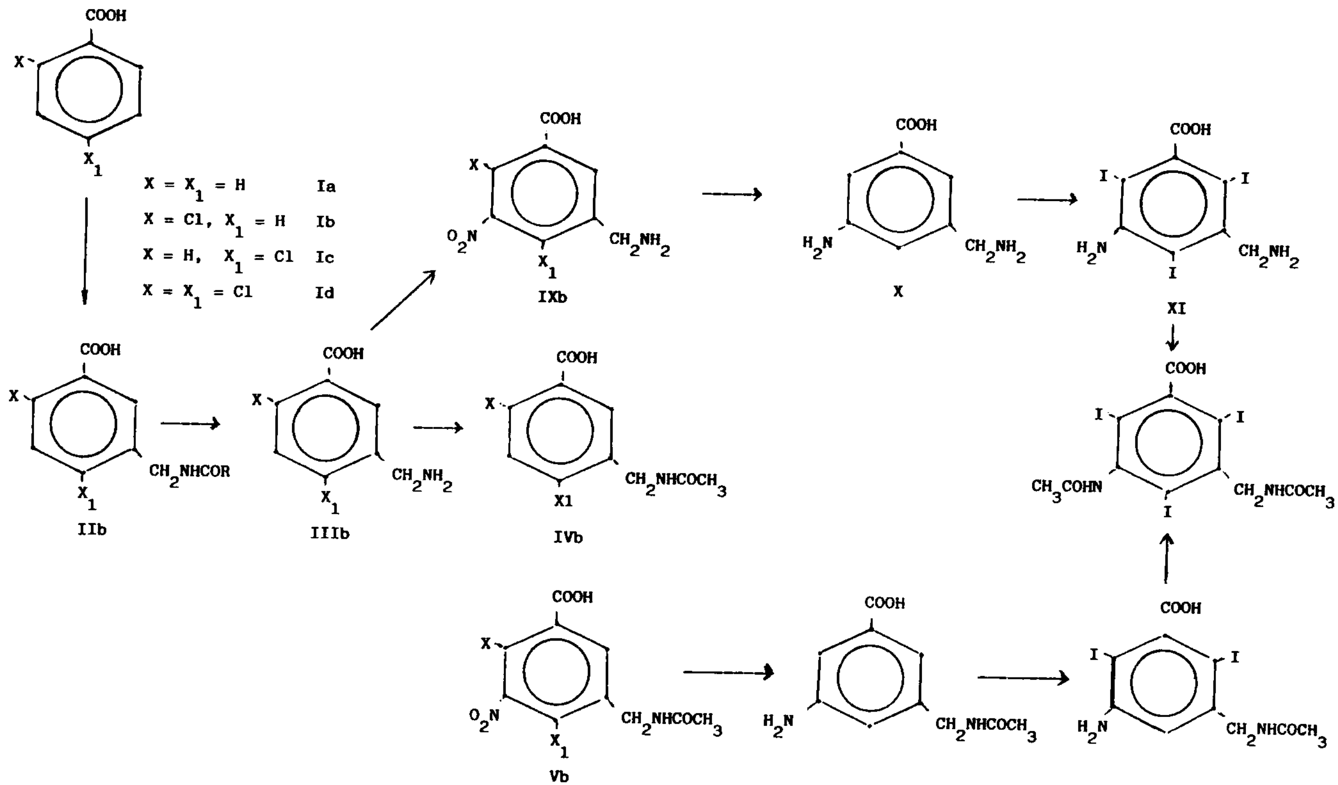


Fig. 4 Synthesis of Iodamide

4. Stability

Iodamide, stored in the solide state at room temperature (15°-25°C) and at 40°C/75%/RH for 12 months, is physically and chemically stable.

Injectable solutions of Iodamide Sodium salt or Meglumine salt stabilized with sodium edetate, have not shown any evidence of chemical change after storage for 60 months at room temperature (15°-25°C).

5. Analysis of impurities

5.1 Free aromatic amine

For the determination of free aromatic amine an official method (2), based on the Bratton-Marshall colorimetric reaction, was reported. The procedure has been automated (9) for bulk product and injectable solutions.

5.2 Free Iodine and free Halides

The method reported by Jap.P. IX for detection of free iodine consists in the extraction with chloroform of a suspension of Iodamide obtained by acidification of an aqueous solution of the sodium salt. Chloroform must be colorless. Free halides are tested with silver nitrate after acidification with nitric acid of an aqueous solution of the ammonium salt of Iodamide and filtration.

5.3 TLC of free amino compounds

The following de-acetylation products may be considered as by-products of Iodamide (A):

3-amino-5-aminomethyl-2,4,6-triiodobenzoic acid (B)
3-amino-5-acetylaminomethyl-2,4,6-triiodobenzoic acid (C)
3-acetamido-5-aminomethyl-2,4,6-triiodobenzoic acid (D)

The Rf values of these compounds in the solvent system n-amylalcohol:acetic acid:water 4:1:5, using Silica-gel 60 F_{254} pre-coated plates (Merck), are summarized in Table 9 (10).

Table 9
Rf of de-acetylation products of Iodamide

Compound	Rf
A	0.14
B	0.08
C	0.3
D	0.03

6. Metabolism and Pharmacokinetics

6.1 Metabolism

Chromatographic studies on urine of normal subjects showed that Iodamide was excreted unchanged in the first 4 hours after administration, as unchanged compound together with amounts of an unidentified metabolite from 4 to 12 hours and mostly as the metabolite after 12 hours. However, the total amount of excreted metabolite is quite small (less than 1.5 per cent of the injected dose) (13).

6.2 Pharmacokinetics

Urinary excretion in rabbits after i.v. administration was found to be (14):

Sodium salt of Iodamide: 84.9% after 195 min.
Meglumine salt of Iodamide: 79.6 after 180 min.

Plasma concentrations in rabbits, after i.v. administration of the meglumine salt of Iodamide, were the following

Minutes	7	10	15	20	27
% dose	42.3±11.3	24.8±9.1	18.1±2.0	11.9±1.9	3.56±1.37

The same Authors report a 97% excretion in 24 hours after i.v. administration to rats and give the content in blood, liver, kidney, spleen and thyroid after 30 min., 1 hour, 2 and 24 hours.

Urinary excretion in mouse is 0.6% after 4 hours when Iodamide is administered orally (300 mg/kg).
As regards humans, urinary excretion after i.v. administration, is of 84.7% after 4 hours and 94.2% after 72 hours while fecal excretion is 0.5%.
Pharmacokinetic parameters for i.v. administration and for infusion are similar and we have $T\frac{1}{2}\beta$ of about 3 min for the distribution half-life and $T\frac{1}{2}\beta$ of about 69 minutes for the disposition half-life (16).

6.3 Protein Binding

Plasma protein binding of Iodamide in 2 patients varied between 4 and 13 per cent (17). Other authors report that the binding to serum protein is negligible (13).

6.4 Acute toxicity

The acute toxicity (DL_{50}) of the meglumine salt of Iodamide are reported in Table 10:

Table 10

Acute toxicity in g/kg for meglumine salt of Iodamide

Animal	i.v.		i.p.	
Mause	9	(16)	11	(18)
Rat	11.4	(16)	17.5	(16)
Rabbit	13.2	(16)	15.5	(16)
Dog	dose of 15 ml/kg of a 50% solution do not cause lethality			

The acute toxicity of the sodium salt of Iodamide in mouse was found to be 17.3 g/kg (19).
H.J. Herms reports a DL_{50} = 16.5 g/kg after i.v. administration of Iodamide (calculated as acid) to rats (19) and on intracerebral toxicity of 0.2 g/kg in mice after administration of the meglumine salt.

7. Polarography

Iodamide exhibits a wave at 0.6 volt in 0.1M phosphate buffer at pH 9.5 (10).

7.1 Elemental composition

Element	% Calc.	% Found
C	22.95	22.93
H	1.77	1.78
I	60.63	60.75
N	4.46	4.42
O	10.19	10.12

7.2 Identification Tests

Iodamide may be identified by the following methods (2) (13):

a) dissolve 0.01 g in 5 ml of hydrochloric acid and heat in a water bath for 5 minutes. The solution must respond to the qualitative test for aromatic primary amines.

b) Heat strongly: violet vapours of iodine are evolved

c) Infrared spectrum recorded on 3 mg, previously dried at 105° for 4 hours, by the potassium bromide disk method exhibits maxima at 3385 cm^{-1}, 1369 cm^{-1}, 1269 cm^{-1}, 1210 cm^{-1} and 1191 cm^{-1}.

d) The UV spectrum of a solution, containing about 10 mg/ml, in a buffer at pH = 9 must show a maximum at 238 nm and $E_{1cm}^{1\%}$ of about 526.

7.3 Official methods

7.3.1 Organically bound iodine

The reductive dehalogenation method with zinc-sodium hydroxide is used to free organically bound iodine. The iodide thus formed is determined by titration in acidic solution with standardized silver nitrate in presence of tetrabromophenolphthalein ethyl ester until the color of the precipitate turns from yellow to green (2).
The determination can be carried out in a simpler way through a reduction with sodium borohydride (10) according to the method first proposed by Egli (11).

7.3.2 Titrimetry

Iodamide can be titrated directly with 0.1N NaOH, after dissolving it in CH_3OH and adding the same volume of H_2O, using phenol red (0.05% of sodium salt) as an indicator, or potentiometrically using a glass electrode (10).

7.4 Chromatography

7.4.1 Paper chromatography

Ascending paper chromatography was performed using Whatman N1 filter paper and the following systems:

a) $n\text{-}C_4H_9OH : CH_3COOH : H_2O = 3:1::2$

b) $CH_3CHOHCH_3 : NH_4OH\ 25\% = 8:2$

The product was visualized as a blue spot on a white background (Rf 0.67 (a), 0.63 (b)/ after spraying with soluble stark, exposing to UV light (254 nm) and again spraying with 2N HCl.

7.4.2 Thin layer chromatography.

Thin layer chromatographic methods for identification and separation of Iodamide and related compounds are summarized in the following Table 10.

Table 10
Thin layer systems of separation of Iodamide (12)

Solvent system	Rf_a	Rf_b
I	0.43	0.59
II	0.50	0.55
III	0.34	0.47
IV	0.70	0.74
V	0.51	0.59
VI	0.44	0.74
VII	0.24	0.33
VIII	0.28	0.50

a) Silica gel 60 F_{254} (Merck) pre-coated TLC plates
b) Cellulose F (Merck) pre-coated TLC plates

- Solvent system:

I = $n\text{-}C_4H_9OH$: CH_3COOH : H_2O - 3:1:1

II = $iso\text{-}C_4H_9OH$: $CH_3CHOHCH_3$: NH_4OH 25% - 2:5:3

III = $iso\text{-}C_4H_9OH$: $CH_3CHOHCH_3$: NH_4OH 25% - 5:2:2

IV = $CH_3COOC_2H_5$: CH_3COOH : H_2O - 3:2:2

V = $n\text{-}C_3H_7OH$: NH_4OH 25% - 7:3

VI = $CHCl_3$: CH_3COOH : H_2O - 5:5:1

VII = $CHCl_3$: CH_3OH : NH_4OH 25% - 6:3:1

VIII = $n\text{-}C_4H_9OH$: CH_3COOH : H_2O - 4:1:5

- Detection system

  a) 0.5% aqueous starch solution with subsequent exposure of the plates to UV light (254 nm): formation of brown spots;

  b) 1:1 aqueous solution of 10% cerium sulfate and 5% sodium arsenite: white spots on a yellow background.

7.4.3 High pressure Liquid Chromatography

A HPLC method for quantitative determination of Iodamide and for separation of possible impurities was developed (10).

Instrumental conditions:

- Apparatus: Hewlett-Packard, mod. 1084B
- Column: 25 cm x 4 mm Hibar(R), packed with Lichrosorb RP-18 (5 μm)
- Injection: 10 μl of a 01 mg/ml aqueous solution (pH 7.5)
- Eluant A: 0.076 M phosphate buffer, pH 7.5, in water
- Eluant B: 1:1 (v/v) methanol:phosphate buffer (pH 7.5)
- Flow: 1.2 ml/min
- Gradient prophile :

minutes	% eluant B
0	15
5	15
15	40
16	40
17	15
22	Stop

- Column temperature: 40°C
- Detection : UV, 240 nm
- t_R : ~ 7.2 min

8. Determination of Iodamide in Body fluids and tissue

The following methods are used for this determination:

a) Total iodine determination, after decomposition with permanganate in hot acid or alkaline medium (16)(23) according to the methods of White and Rolf (22);

b) Total iodine (13) (14) determination with a PBI Technicon Autoanalyzer (23);

c) Spectrophotometric determination in diluted urine of dog (6);

d) X-Ray Fluorescence analysis after irradiation with ^{241}Am (15);

e) Total radioactivity of ^{131}I material determined in a gamma counter (17).

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LITHIUM CARBONATE

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ANALYTICAL PROFILES OF DRUG SUBSTANCES
VOLUME 15

LITHIUM CARBONATE

1. Description

1.1 Name, Formula, Molecular Weight

Chemical Name
Lithium Carbonate

Nomenclature
The following nomenclature is used in Chemical Abstracts: Carbonic Acid, Dilithium Salt [554-13-2]

Trademarks
The following trademarks are listed in the Merck Index (1): Camcolit; Candamide; Carbolith; Ceglution; Eskalith; Hypnorex; Lithane; Lithobid; Lithonate; Lithotabs; Plenur; Priadel; Quilonum retard.

Molecular Formula and Weight (1)
Li_2CO_3 73.89
C: 16.25% Li: 18.78% O: 64.96%

1.2 Appearance, Color, Odor

White, granular, odorless, light alkaline powder (1,2).

1.3 Drug Properties

Lithium Carbonate, by virtue of the therapeutic properties of lithium, is used for the treatment of manic depressive psychoses. The drug is listed in the United States Pharmacopea (2), the British Pharmacopoeia (3), and the Modern Drug Encyclopedia (4), as well as Remington's Pharmaceutical Sciences (5).

2. Physical Properties

2.1 Infra-red Spectroscopy

The infra-red spectrum of lithium carbonate dispersed in KBr is shown in Figure 1 (6). Literature sources have demonstrated that the infra-red spectrum of lithium carbonate is consistent with its known crystal structure (7, 8). The infra-red spectrum of lithium carbonate

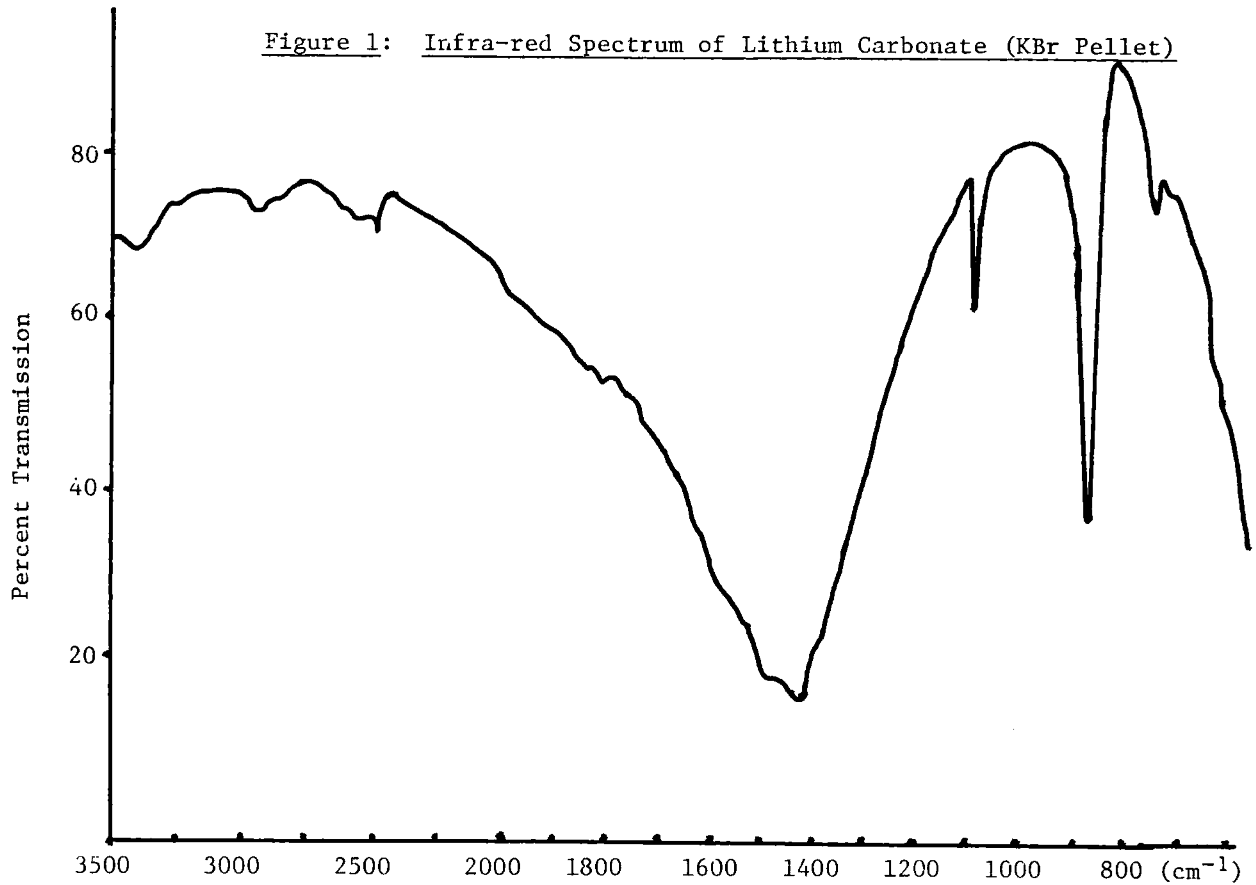


Figure 1: Infra-red Spectrum of Lithium Carbonate (KBr Pellet)

has also been obtained as a film on sodium chloride plate and as Vaseline and flurolub suspensions (7, 9). A summary of the characteristic infra-red bands for lithium carbonate is presented in Table I.

TABLE I

Characteristic Infra-red Bands of Lithium Carbonate (7)

Frequency (cm^{-1})	Wavelength (μ)	Relative Intensity
2558	3.91	W
2494	4.01	W
1842	5.43	W
1806	5.54	W
1495	6.69	VS
1437	6.96	VS
1088	9.19	M
866	11.55	S
846	11.82	W
741	13.50	W
712	14.04	VW

The infra-red absorption band at 1088 cm^{-1} can be used to uniquely quantitate lithium carbonate in the presence of other alkali carbonates (10). The observed frequencies related to the isotopic species $^6Li_2CO_3$ and $^7Li_2CO_3$ have been reported by Tarte (11).

2.2 Raman Spectroscopy

The Raman spectra of crystalline and molten lithium carbonate have been reported by Brooker and co-workers (12, 13). Major bands are observed at 1091 and 1459 cm^{-1}.

2.3 Atomic Emission and Absorption Spectroscopy

Lithium carbonate can be made to exhibit the characteristic emission spectrum of lithium. Three typical analytical emission lines are obtained for lithium containing aqueous solutions. These are summarized along with their relative intensities in Table II (14).

The line at 670.8 nm is particularly intense and imparts a deep red color to an oxidizing flame.

TABLE II

Lithium Analytical Emission Lines

Wavelength (nm)	Relative Intensity*
670.8	1
323.3	235
610.4	3600

*relative amount of lithium required for 1% response

Typical sources of excitation include air-acetylene and nitrous oxide-acetylene flames. More recently electrothermal and argon plasma excitation techniques have become available. Because of its greater sensitivity the line at 670.8 nm is most often used for the analysis of lithium by flame emission spectroscopy, atomic absorption spectroscopy and plasma spectroscopy (14, 15).

2.4 Melting Point

The melting behavior of lithium carbonate has been evaluated by DTA using both heating and cooling programs. Lithium carbonate has been reported by various sources to melt in the temperature range of 714° to 733°C (16-25) depending on the atmosphere employed (ie. CO_2 or air) and the degree of dissociation of Li_2CO_3 to Li_2O and CO_2 that occurs as the melting point is approached (16, 18, 20, 21).

Typical thermal properties reported in the literature for lithium carbonate are summarized in Table III.

TABLE III

Thermal Properties of Lithium Carbonate

Melting Point (23)	993°K; 720°C
Heat Capacity (23)	23.2 cal/mole /deg
Specific Heat (24)	0.315 cal/g
Heat of Fusion (24)	10.7 kcal/mole

2.5 Thermogravimetric Analysis

Data obtained for a typical lot of lithium carbonate (H_2O < 0.5%) by thermogravimetric analysis is presented in Table IV. Under the conditions employed the compound is essentially weight stable up to 200°C with only water loss. Above 200°C a gradual continuing weight loss is observed. This behavior is consistant with that reported by Machaladze and co-workers (21).

TABLE IV

Lithium Carbonate: Thermogravimetric Behavior (26)

TGA (N_2 atmosphere)	Perkin-Elmer TGS-1
Scan rate 10°C/minute	
RT to 90°C	0.11% weight loss
90° to 200°C	0.08% weight loss
200° to 450°C	0.63% weight loss
above 450°C	continuing weight loss

2.6 Dissociation Constant

The pKa's for the first and second ionization steps of the conjugate acid of the carbonate ion are reported in the literature to be 6.38 and 10.25 respectively (27).

2.7 <u>Conductivity</u>

The relationship between the equivalent conductance (Λ) and the concentration of lithium carbonate is typical of a strong electrolyte. A plot of Λ versus $\sqrt{C}$ yields straight line for concentrations less than 0.01N. The equivalent conductance at infinite dilution (Λ_0) for lithium carbonate was determined to be 110.2 $\Omega/cm^2/Eq$ at approximately 25°C, from this plot (6).

2.8 <u>Microscopy</u>

USP Lithium Carbonate is a microcrystalline solid that is birefringent under crossed polars. The solid has been observed to exist as polycrystalline aggregates ranging from approximately 10 to 85 micrometers in diameter (6).

2.9 <u>Index of Refraction</u> (N_D^{25})

The refractive indices of Lithium Carbonate have been reported as 1.428, 1.567 and 1.572 (25). Lithium Carbonate is biaxial and is optically negative.

2.10 <u>Density</u>

The density reported for Lithium Carbonate is 2.11 g/cc (25).

2.11 <u>Crystal Structure</u>

The crystal structure of Lithium Carbonate is monoclinic with unit cell dimensions of a = 8.39 Å, b = 5.00 Å, c = 6.21 Å, and β = 114.5± (28). The crystal lattice belongs to the space group C_{2h}^{6} - C 2/C and there are four lithium carbonate molecules per unit cell (29, 7).

2.12 <u>X-Ray Powder Diffraction</u>

Major lines present in the x-ray powder diffraction pattern of USP grade lithium carbonate are presented in Table V. Strong lines are observed at 31.6, 21.4, and 30.6 degrees 2θ for copper $K\alpha$ radiation. These values are in good agreement with the literature values of 2θ = 31.4, 21.0 and 30.3 [ASTM Data (30)] and 2θ = 31.8, 21.3, and 30.6 [JCPDS Data (31)].

Typical instrumental and experimental conditions used to obtain the x-ray powder diffraction pattern of lithium carbonate depicted in Figure 2 (32) are presented below.

Instrumental Conditions

Spectrometer:	Diano 8535 Diffractometer
Generator:	30 KV, 13 mA
Tube Target:	Cu
Radiation:	Cu, Ni Filtered, $K\alpha$ = 1.542 Å
Optics:	1° Beam Slit, MR Soller Slit, 0.1° Detector Slit, 3° Take-Off Angle
Goniometer:	Scan Rate: 2 degrees 2θ/minute
Detection:	SPG-10 Detector Rate meter 2500 cps full scale Pulse Height Selection, E_L = 0.2V ΔE = 10
Sample Preparation:	Sample was ground, sieved through a No. 100 US Standard Sieve, and back-packed into an aluminum sample holder.

X-Ray Powder Diffraction Pattern of Lithium Carbonate (USP)

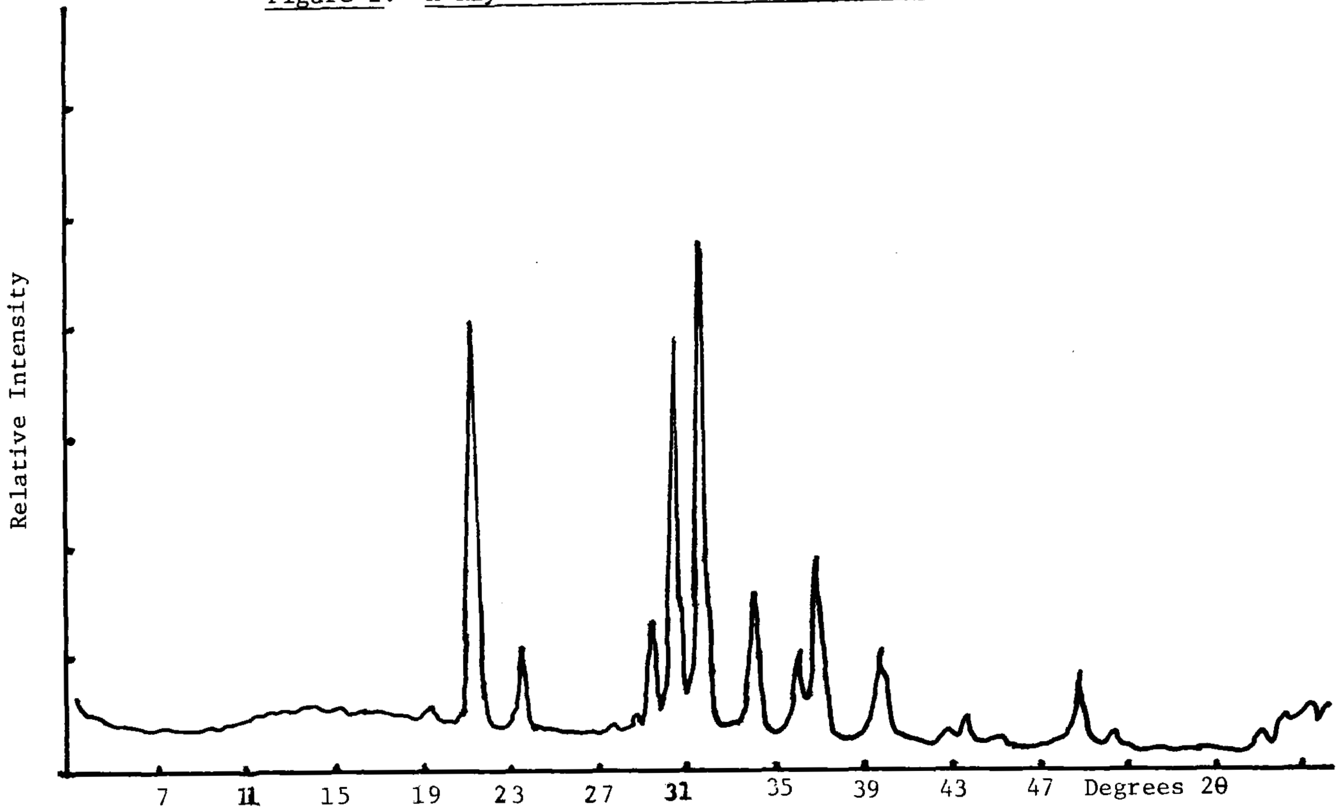
MAJOR LINES

2θ Degrees *	d **	I/Io ***
21.4	4.15	84
23.5	3.79	18
29.5	3.03	24
30.6	2.92	81
31.6	2.83	100
34.1	2.62	31
36.1	2.49	19
36.9	2.43	40
39.7	2.27	19
48.8	1.87	15

* 2θ degrees read to nearest 0.1 degrees 2θ

** Interplaner Distance (Å): $d = \frac{n\lambda}{2 \sin \theta}$

Figure 2: X-Ray Powder Diffraction Pattern of Lithium Carbonate



*** Relative Intensity in percent based on strongest signal. Under the experimental conditions employed the relative intensities are subject to change due to variations in sample handling and particle size and are only included as a guide for identifying strong lines.

The Hanawalt indices for lithium carbonate are 2.81_x, 4.16_8 and 2.92_8 (33).

2.13 Polymorphism

Recent literature indicates that up to its fusion temperature lithium carbonate exists as only one crystal form (18). An earlier report in the literature indicated the presence of a polymorphic transition at 166°C (17). The possibility of lithium carbonate existing as a stable α and a metastable β phase, under atmospheric conditions, also has been proposed (34). It should be noted, however, that in the crystalographic sources consulted (30, 31, 33) only one form of lithium carbonate is listed, implying that other crystalline forms are uncommon.

Impurities present in lithium carbonate may account for some of the thermal effects noted. In this respect, Reisman reported an anomalous transition at ~410°C and an additional heat effect at 350°C (18). The later was suspected to be due to the presence of Li_2O. Impurities such as Li_2O, Na_2CO_3 and K_2CO_3 are known to form eutectics with lithium carbonate and may account for some of the anamolies observed. The ternary eutectic with Na_2CO_3 and K_2CO_3 melts at 397° (19).

2.14 Solubility

The following equilibrium solubility data was obtained either experimentally at 37°C (6) or from the literature as indicated in Table VI.

TABLE VI

Solubility of Lithium Carbonate in Common Solvents

Solvent	Solubility (g/100 ml)	Source
Water, 0°C	1.5	(25)
Water, 37°C	1.0	experimental
Water, 100°C	0.7	(25)
0.1N NaOH, 37°C	1.1	experimental
1.0N NaOH, 37°C	1.7	experimental
0.05M tris-buffer, 37°C	1.1	experimental
Ethanol	Insoluble	(25)
Acetone	Insoluble	(25)

Lithium carbonate decomposes in strong mineral acids to yield carbonic acid, carbon dioxide and the conjugate salt. The solubility of lithium carbonate in water has been extensively studied as a function of temperature and has been observed to have a negative temperature coefficient of solubility; the solubility decreasing significantly with increasing temperature (35, 36). The heat of solution of lithium carbonate in water at 25°C has been reported as -14,800 ±0.021 kJ mol^{-1} (37).

When considering the solubility of lithium carbonate in aqueous solutions, it should also be noted that lithium forms insoluble salts with several common anions including phosphate, fluoride and the carboxylate anion of the C_{14} -C_{18} fatty acids (25).

The solubility product of lithium carbonate in water at 25°C is 1.7 x 10^{-3} (38) when the concentration of the lithium and carbonate ions are expressed in moles/liter.

2.15 Dissolution

The intrinsic dissolution rate of lithium carbonate in aqueous solutions was reported by Wall and co-workers (39) using the rotating disc method. They found linear dissolution rate profiles for

lithium carbonate in water, simulated gastric fluid and tris buffer. Dissolution studies in simulated intestinal fluid containing phosphate were complicated by the precipitation of trilithium phosphate onto the disc.

Dissolution rate determinations for various experimental and commercial lithium carbonate preparations have been reported in the literature (40 - 44). Ritschel and Parab (43) evaluated seven (six conventional and one sustained release) lithium carbonate commercial preparations. For the conventional preparations they found a good correlation between the ENSLIN number and t (5 min.), t (10 min.) and MRT (mean residence time). The ENSLIN number (amount of water in mL absorbed by 1 g of powdered substance) is a measure of the hydrophilicity, or wetting, of the formulation.

The wetting of pharmaceutical powders, including lithium carbonate, has also been evaluated by Lerk and co-workers (45) using contact angle measurements. The contact angle θ obtained for lithium carbonate by these workers was 50° indicating hydrophilicity.

3. Preparation of Lithium Carbonate

Lithium carbonate is primarily prepared from the mineral spodumene, $LiAlSi_2O_6$ (46). Other mineral sources of lithium include petalite ($LiAlSi_4O_{10}$), amblygonite ($LiAl[F,OH]PO_4$) and lepidolite ($K_2Li_3Al_4Si_7O_2[OH,F]_3$). Spodumene is the most commercially important of the lithium ores because of its relative abundance and its relatively high lithium content (3.75%).

In the manufacturing process employed by the Lithium Corporation (47) spodumene crude ore, which also contains such components as mica, quartz and feldspar, is crushed to a fine sand. The crushed spodumene is separated from the other components by flotation. The "purified" spodumene is subjected to intense heat (approximately 1100°C) and milled to a fine powder to increase its surface area and reactivity. The powdery

spodumene is treated with strong sulfuric acid at 250°C to produce lithium sulfate (Li_2SO_4). The lithium sulfate produced is separated from the residual insoluble components of the ore by aqueous dissolution. The resulting lithium sulfate solution is reacted with sodium carbonate to produce lithium carbonate, which remains in solution. Impurities yielding insoluble carbonates are precipitated in this step. The lithium carbonate solution is further purified by pH adjustment and filtration and concentrated by evaporation. Lithium carbonate is precipitated from the concentrated solution by further treatment with sodium carbonate. Pharmaceutical grade material is also further processed to meet compendial and special requirements such as particle size and bulk density. The flow chart depicted in Scheme I summarizes the process just described.

Other processes reported to utilize spodumene involve treatment with limestone to produce lithium hydroxide (46) and direct recovery of lithium carbonate by digestion with aqueous sodium carbonate at 200°C (48). The production of lithium carbonate from lithium hydroxide has been accomplished using carbonization (CO_2) (49) and treatment with urea (50).

A procedure for purifying lithium carbonate by suspension in boiling water is described in Volume I of "Inorganic Synthesis" (51). The procedure is based on the fact that lithium carbonate is less soluble in hot water than in cold water, in contrast to the salts that are present as impurities. A zone melting procedure for producing single crystals of lithium carbonate has also been described (52).

4. Stability

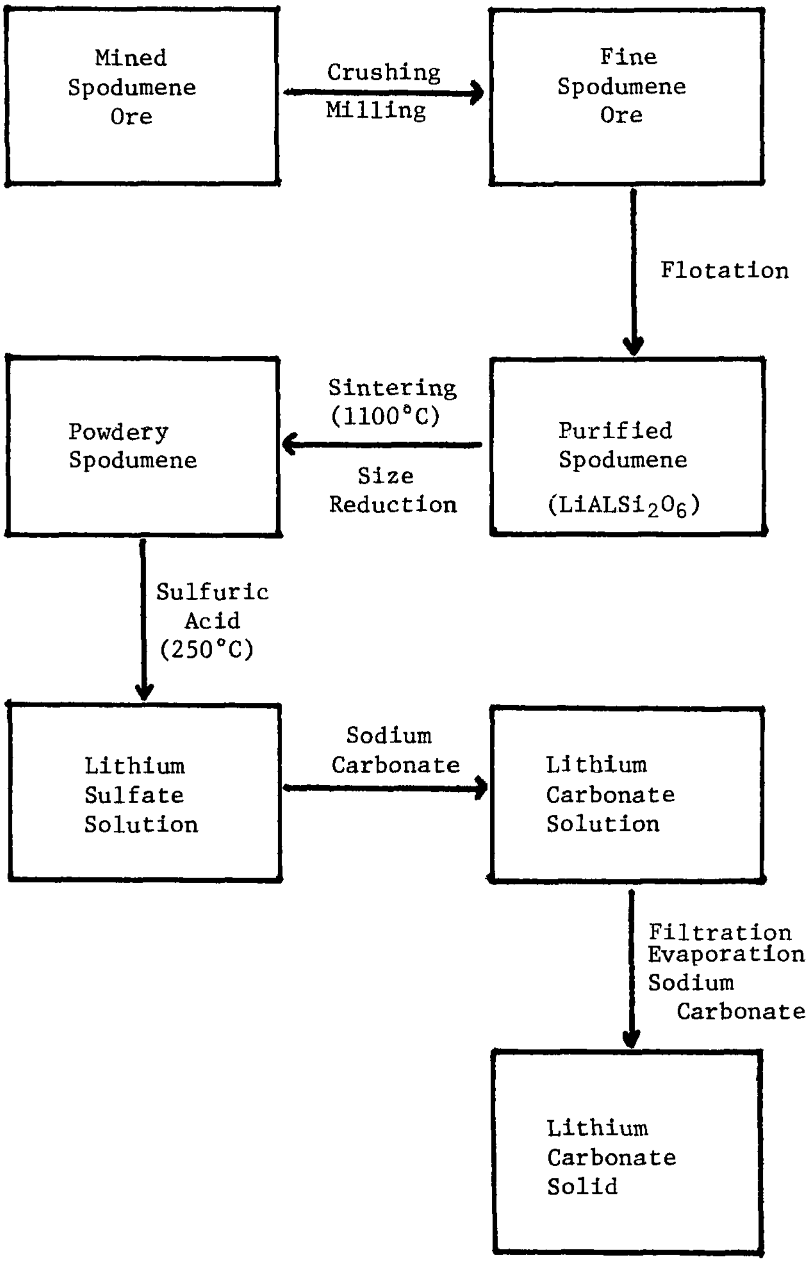
4.1 Solution

The predominant stability problem for lithium carbonate in aqueous solutions occurs in acid solutions, in which it decomposes to yield the lithium salt of the acid, bicarbonate, carbonic acid and ultimately, carbon dioxide.

Solutions of lithium carbonate are also incompatible with a variety of cations and anions.

SCHEME I

Preparation of Lithium Carbonate from Spodumene



Cations such as calcium and barium, whose carbonate salts are much less soluble than lithium carbonate, and anions such as phosphate, that form less soluble lithium salts, should be excluded from lithium carbonate preparations.

4.2 Solid State

Light

Pharmaceutical grade lithium carbonate was subjected for one week to visible light, whose intensity was 600 foot candles (6). No decomposition as noted by changes in physical appearance (color, texture), weight and titrimetric assay for carbonate was observed.

Thermal

Samples of lithium carbonate were stored in open weighing dishes at 25°C and 105°C, respectively, for one week (6). A small increase in the titrimetric assay for carbonate from 99.4% to 99.6% was observed for the sample stored at 105°C. No change in the assay was noted for the sample stored at 25°C. No measureable changes in weight or physical appearance were observed for either sample. This stable behavior for lithium carbonate in the solid state is confirmed by the literature (16, 17, 21). Accordingly, the lowest temperature at which lithium carbonate has been reported to begin to dissociate to lithium oxide and carbon dioxide is 200°C (21). Other workers have reported that dissociation occurs only near the melting temperature (15, 16).

Humidity

Samples of lithium carbonate stored for one week at 25°C/85% RH, 35°C/10% RH and 35°C/85% RH were essentially weight stable (6). This is consistent with the literature (8, 10), which indicates that lithium carbonate is not very hygroscopic.

5. Methods of Analysis

5.1 Identification Test for Lithium (2)

When lithium carbonate is moistened with hydrochloric acid, it imparts an intense crimson color to a non-luminous flame.

5.2 Identification Test for Carbonate (2)

Lithium carbonate effervesces upon the addition of an acid, yielding a colorless gas, which when passed into a solution of calcium hydroxide, immediately causes a white precipitate to form.

5.3 Microchemical Test for Lithium

The microscopic identification of lithium is practical only in materials in which lithium is present in relatively high concentrations. Identification has been achieved by preparation of the tri-lithium phosphate salt, which forms star-like clumps (53), the pyroantimonate salt, which gives hexagons (53) and an orange-brown aurate salt (54). In the absence of other alkali metals lithium reacts with zinc uranylacetate to yield regularly developed octahedra (55).

5.4 Microchemical Test for Carbonate

Absorption of carbonate, evolved upon acidification of a lithium carbonate preparation, by a hanging drop of lead acetate, gives rise to acicular crystals occurring singly or in irregular aggregates. Both bicarbonate and carbonate give a positive response under these conditions. When added directly, thallus acetate does not precipitate with bicarbonate, but forms colorless, long, slender needles with carbonate (56).

5.5 Volumetric Analysis

Lithium carbonate is analyzed in the USP (2), BP (3) and ACS Reagent Chemicals (57) by titration of the carbonate anion. Excess strong acid is added to a solution of lithium carbonate and the residual acid remaining after neutralization is back-titrated with sodium hydroxide. Differences exist between these methods with respect to the indicators used and the emphasis placed on expulsion of carbon dioxide after acidification. Alternatively, potentiometric end point detection can be accomplished using glass and calomel electrodes.

5.6 Atomic Emission and Absorption Spectroscopy

Solutions of lithium carbonate are frequently analyzed for lithium using emission techniques such as flame photometry, plasma spectroscopy and by atomic absorption. The methods typically employ analysis at a wavelength of 670.8 nm, which is the most sensitive specific lithium line (2, 14). These techniques are particularly effective for the analysis of lithium in dosage forms and biological fluids (2, 40, 44, 58). A detailed discussion of the analysis of lithium in biological fluids and tissues by flame photometry and atomic absorption is presented in the text "Lithium Research and Therapy" (59).

5.7 Other Spectrometric Techniques

Lithium carbonate has been determined spectrophotometrically in tablets using the color formed upon complexation of lithium with a "crowned" dinitrophenylazophenol ether (60). The purple color produced is stable and the absorbance of the solution measured at 560 nm is linear over the concentration range from 25 to 250 ppb. Field desorption mass spectrometry (FD-MS) in conjunction with a multichannel analyzer has proven to be a useful tool for trace analysis of lithium (61). The method also allows for the determiation of the isotopic distribution of the lithium salt analyzed.

5.8 Conductivity

Although nonspecific, conductimetric methods have been used for the analysis of strong electrolytes (62). The methods tend to be easily automated. The technique is comparatively simple and applicable to the dissolution rate determination of dosage forms providing that the other conducting components of the formulation are not present in significant amounts. It has been used successfully in the authors laboratory for the determination of the dissolution rate of lithium carbonate sustained release formulations in water (63). Precautions were required against absorption of atmospheric CO_2. This was accomplished by performing the dissolution rate studies with the vessels under a steady stream of nitrogen.

5.9 Ion Selective Electrodes

Several workers have explored the usefulness of ion selective electrodes for the analysis of solutions of lithium (64, 65, 66). The electrodes are limited by comparatively poor Li+/Na+ selectivity. This deficiency is especially apparent in biological fluids where comparatively high levels of Na+ are encountered. Using elecrodes based on PVC membranes containing ETH 1810 as a neutral carrier, Metzger and co-workers (64) obtained recoveries of lithium in serum to within ±10% over the clinically relevant concentration range.

5.10 Ion Chromatography

Aqueous solutions of lithium carbonate can be readily analyzed for lithium by ion chromatography. A polystyrene-divinyl benzene sulfuric acid cation exchanger in the hydrogen form (Waters, IC-PAC-C) and 2 mM nitric acid eluent is employed (67). Under these conditions lithium at the ppm level is readily separated from sodium and potassium at comparable concentrations. The carbonate anion can also be determined using a polymethacrylate anion exchanger in the quaternary ammonium ion form (Waters, IC-PAC-A).

6. Medicinal History (68, 69)

Lithium, the lightest of the alkali metals, was discovered in 1817 by Arfwedson. Its salts were later found in the spa waters of Germany and England, where it was believed that it was therapeutic in the treatment of rheumatoid arthritis and gout. Its usefulness in the treatment of gout was attributed to the high solubility of lithium urate. The use of lithium as a uricosuric prompted J. F. Cade in Australia to give animals a lithium salt to decrease the nephrotoxicity of uric acid. He noted that the lithium salt produced a calming effect in the animals and proceeded to use lithium salts clinically as a sedative. During the 1950's and 1960's the clinical use of lithium

salts was intensely investigated in Europe, where it became accepted as an effective and safe treatment for manic depressive illness.

Lithium salts were not accepted in the United States until 1970. This was due in part to concerns about the safety of lithium salts after several deaths were reported (1949-50) among patients using large amounts of lithium chloride as a salt substitute. Although several lithium salts such as the chloride and bromide have been used, lithium carbonate is preferred because of its relative stability (it is less hygroscopic than the halogen salts) and it is less irritating to the gastrointenstinal tract.

7. Pharmacology (69, 70)

Lithium carbonate is usually administered as 300 mg tablets or capsules. Each 300 mg of the carbonate contains 8.12 mEq of lithium. The usual daily dose of lithium carbonate for prophylactic therapy is 600 mg to 1200 mg.

Lithium is readily absorbed after oral administration in the gastrointestinal tract, peaking in the plasma within 1 to 3 hours and tends to distribute evenly throughout the total body water space. Lithium is not bound to plasma proteins. There is some lag in penetration into the cerebrospinal fluid, but there is no absolute barrier to its entry into the brain. Equilibration of lithium between the blood and the brain is almost complete within 24 hours.

The "metabolism" of the lithium ion is almost entirely via the kidneys. About half of a single dose of lithium is excreted in 24 hours. An important feature of the renal excretion of lithium is that its rate is not typically increased by the administration of most diuretics. Increased administration of sodium appears to have minimal effect on the normal excretion of lithium, while depressed in-vivo levels of sodium facilitate lithium retention. These physiological factors have important implications for the management of lithium intoxication. The therapeutic plasma range (0.5 - 1.5 mEq/L) and the toxic plasma levels (>1.6 mEq/L) are very close and monitoring is performed at

the onset of therapy. The mechanism of action of lithium ion in manic depression is not clearly understood. Lithium interferes with the action of the catecholamines in the brain. This supports the popular hypothesis that in mania catecholamines may be functionally overactive in the brain. The role played by lithium may also be related to competition with sodium ions in body sites, such as electrolyte balance across cell membranes, including those of the neurons.

Acknowledgement
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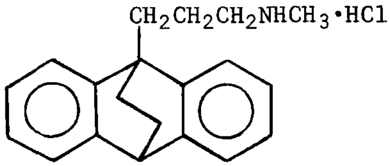
MAPROTILINE HYDROCHLORIDE

Soonhee K. Suh and James B. Smith

1. Description

1.1 Name, Formula, Molecular Weight

Maprotiline hydrochloride is 1-(3-methylamino-propyl)-dibenzo[b,e]bicyclo[2.2.2.]octadiene hydrochloride.



$C_{20}H_{23}N \cdot HCl$ Molecular Weight 313.87

1.2 Appearance

Maprotiline hydrochloride occurs as a white to off-white, odorless, fine, crystalline powder.

2. Physical and Chemical Properties

2.1 Infrared Absorption Spectrum

The infrared absorption spectrum obtained from a mineral oil dispersion of maprotiline hydrochloride on a Perkin Elmer Model 281B IR spectrophotometer is shown in Figure 1. The spectral assignments are listed in Table I.

Table I

Wavenumber, cm^{-1}	Assignment
2720-2780	$N-CH_3$
2455	$-NH_2-$
1596	Aromatic stretch
746-755	o-substituted benzene ring

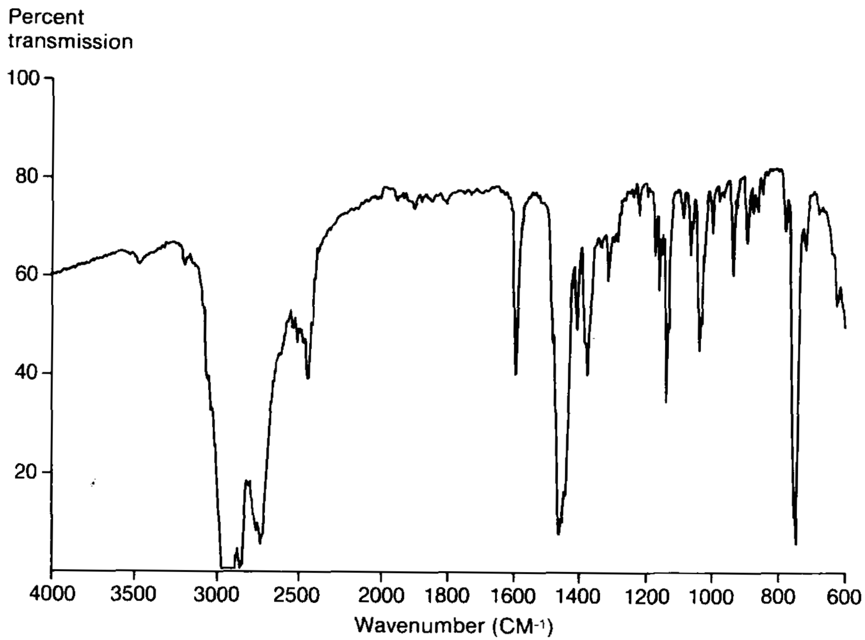


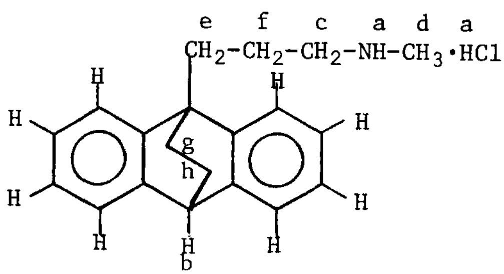
Figure 1
Infrared Absorption Spectrum of Maprotiline Hydrochloride

2.2 Nuclear Magnetic Resonance Spectrum (NMR)

The ^{1}H-NMR spectrum of maprotiline hydrochloride is shown in Figure 2. The spectrum was determined on a Varian CFT-20 NMR instrument at ambient temperature. The sample was dissolved in deuterated dimethylsulfoxide containing tetramethylsilane as an internal standard. The spectral assignments are shown in Table II.

Table II

Chemical Shift (ppm)	Multiplicity	No. of Protons	Assignment
1.65	m	4	g, h
2.45	m	4	e, f
2.66	s	3	d
3.19	t	2	c
4.24	t	1	b
7.0 - 7.4	m	8	aromatic H
9.7	s (broad)	2	a



2.3 Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum of maprotiline hydrochloride in methanol (0.1 mg/ml) exhibits two maxima at 265 and 272 with A(1%,1cm) values of 40.0 and 49.7, respectively. A typical spectrum obtained from HP 8450A UV/Visible spectrophotometer is shown in Figure 3.

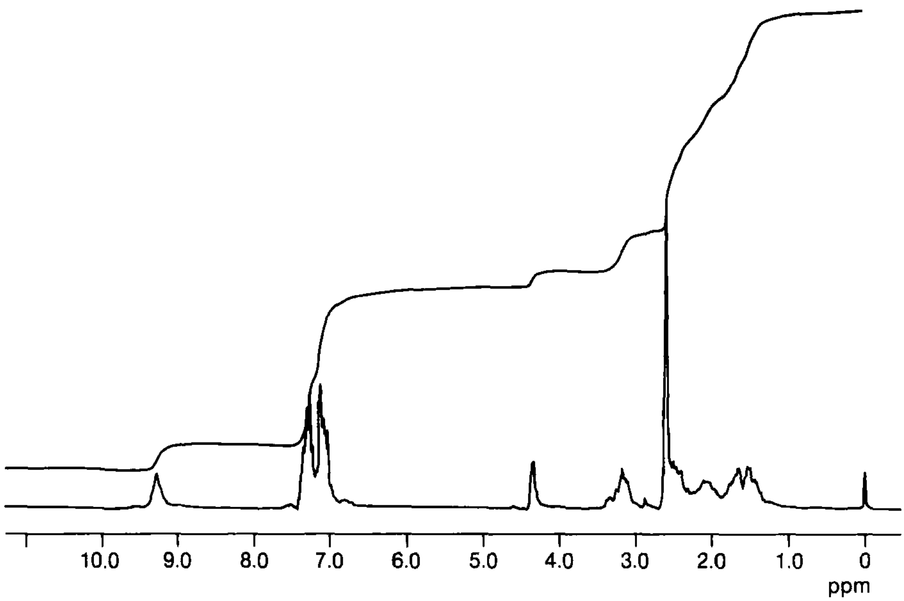


Figure 2
NMR Spectrum of Maprotiline Hydrochloride

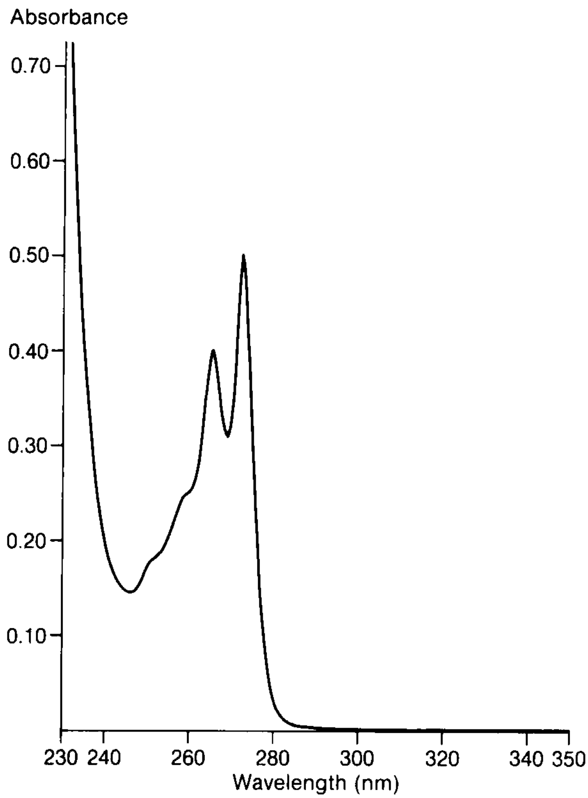
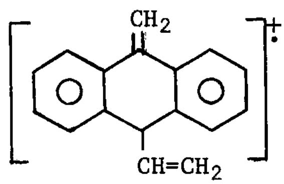
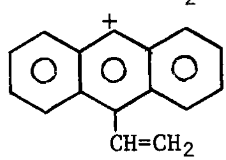
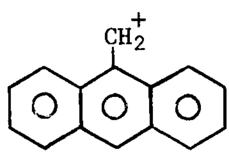
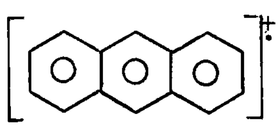


Figure 3
Ultraviolet Absorption Spectrum of Maprotiline Hydrochloride

2.4 Mass Spectrum

Figure 4 shows the mass spectrum of maprotiline hydrochloride obtained at 70 ev on a Kratos MS25 spectrometer using solid probe insertion. An interpretation of the fragmentation ions is given in Table III.

Table III

m/z	Species
277	M^{+}
247	$M - NHCH_3$
218	
203	
191	
178	
70	$CH_2{=}CH{-}CH{=}N^{+}H{-}CH_3$
44	$CH_2{=}N^{+}H{-}CH_3$

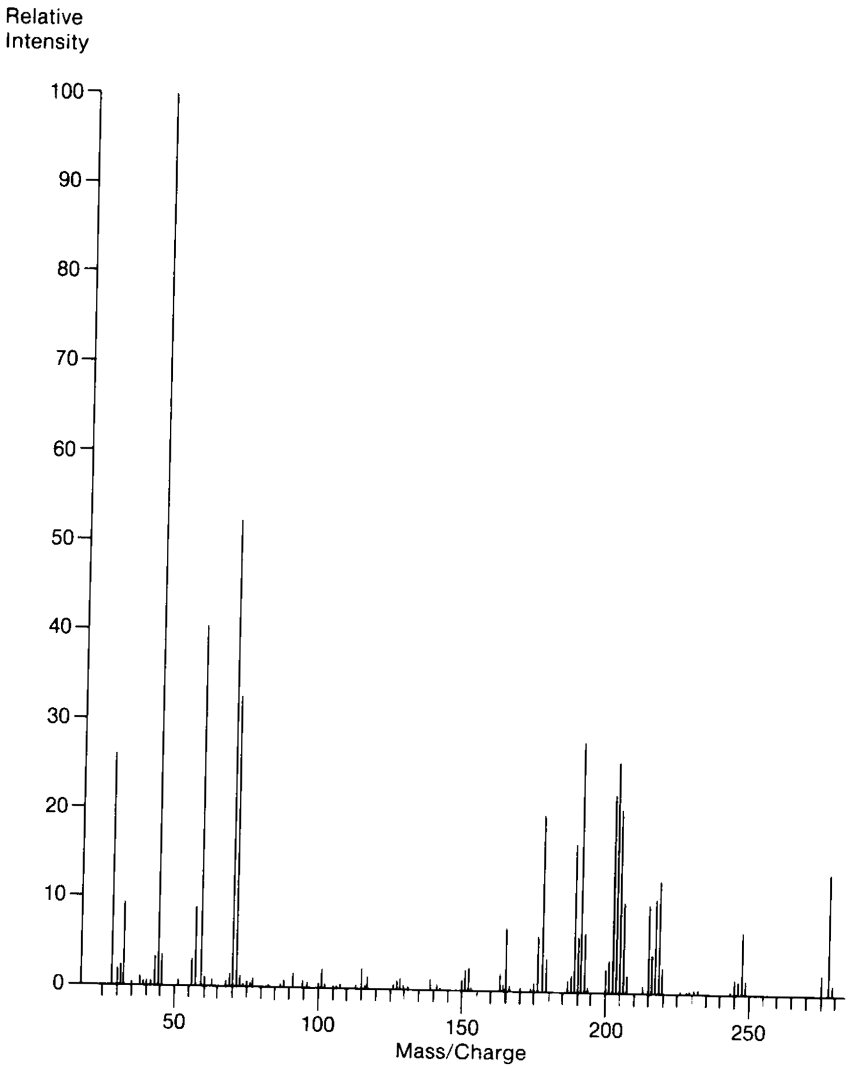


Figure 4
Mass Spectrum of Maprotiline Hydrochloride

2.5 Polymorphism

Maprotiline hydrochloride is polymorphic and known to exhibit several crystal forms (1, 2, 3). A typical production batch is a mixture with varying ratios of crystal form A and B. The two crystal forms show no difference in solubility characteristics at room and physiological temperatures. In the solid state, they show differences in IR, DSC and x-ray powder diffraction patterns. Crystal form A is thermodynamically stable under 104°C, while the B form is metastable. Under conditions such as storage and tablet compression, no transformation of form B to form A, or the reverse, has been observed. However, a transformation of form B to form A has been observed upon melting.

2.6 Melting Range

Maprotiline hydrochloride melts between 237 - 246°C with partial decomposition.

2.7 Differential Scanning Calorimetry (DSC)

The existence of polymorphism has also been shown by differential scanning calorimetry. A typical batch of maprotiline hydrochloride frequently exhibits two melt endotherms indicating the presence of two crystal forms. The thermogram shown in Figure 5 was produced on a Perkin Elmer DSC-2, scanning 10°C per minute. Crystal forms A and B show extrapolated melting points at 242°C and 239°C, respectively.

2.8 X-Ray Diffraction

The x-ray powder diffraction pattern obtained for a typical batch of maprotiline hydrochloride, as shown in Figure 6, is relatively weak with the strongest reflection occurring at approximately 16.4 ± 0.2 degrees 2θ. The characteristic reflection patterns attributable to crystal form A and B, although very weak signals, appear at 3.85 and 3.60 angstrom, respectively. They are the basis for quantitative determination of a mixture by x-ray diffraction (4).

2.9 Thermogravimetric Analysis

The TGA of maprotiline hydrochloride typically exhibits a mean weight loss of about 0.2% between

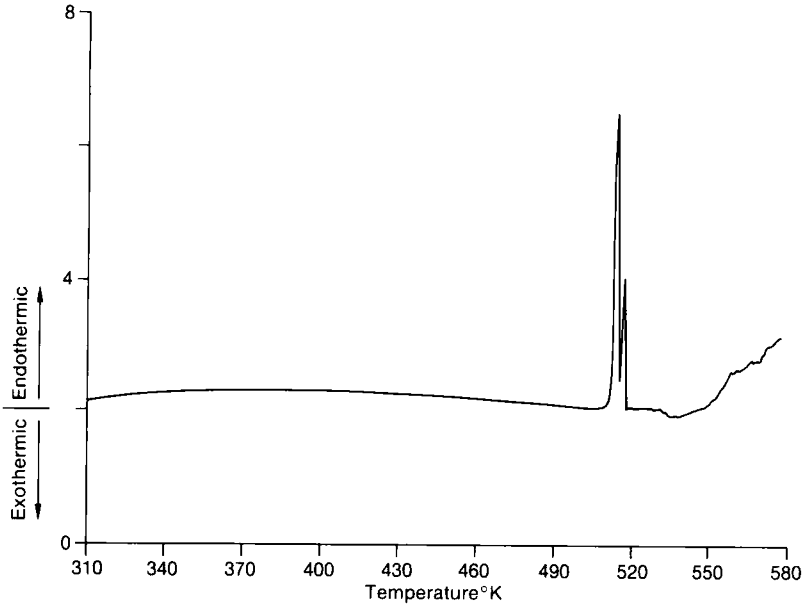


Figure 5
DSC Scan of Maprotiline Hydrochloride

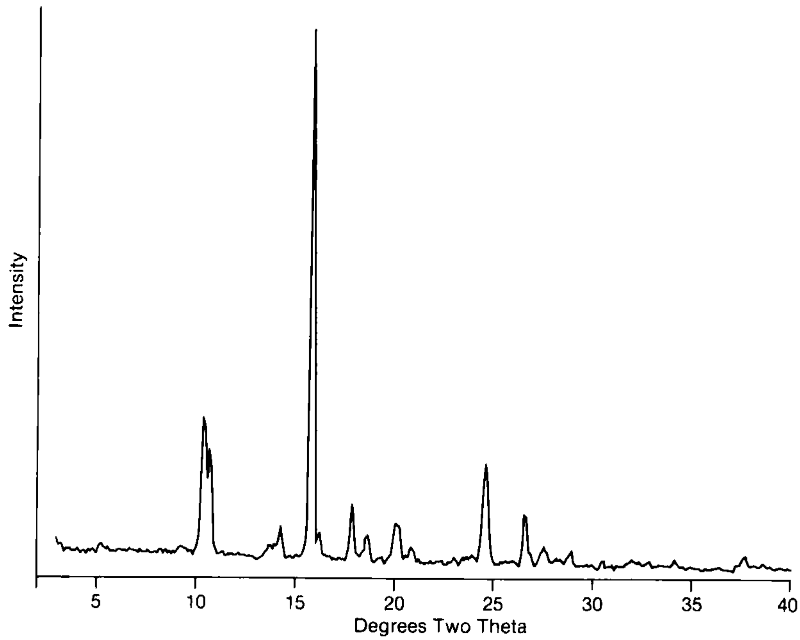


Figure 6
X-ray Powder Diffraction Pattern of Maprotiline Hydrochloride

room temperature and 175°C. Above 175°C a rapid weight loss due to decomposition and/or sublimination is observed.

2.10 Dissociation Constant

The pKa value determined in water at 25°C is 10.5 ±0.2 (5). Also, values of 9.0 ± 0.1 and 9.4 ± 0.1 determined in 80% ethylene glycol monomethyl ether in water have been reported (1, 6).

2.11 Solubility

Maprotiline hydrochloride is freely soluble in methanol and chloroform, slightly soluble in water, and practically insoluble in isooctane. Solubilities in some commonly used organic solvents and aqueous solutions are shown in Table IV.

Table IV

Solvent	T (°C)	pH	Solubility (mg/ml)	Reference
Chloroform	25	--	100	7
Chloroform	30		142	9
Methanol	25	--	100	7
Dimethylformamide	25	--	30	7
Acetone	25	--	0.1	7
Ether	25	--	0.02	7
Water	25	5.6	10.3	8
Water	30	5.7	11.1	9
Water	37	5.6	13.2	8
0.1N HCl	25	1.0	3.1	8
0.1N HCl	37	1.0	4.4	8
Gastric Fluid (simulated)	37	1.1	3.7	1
Intestinal Fluid (simulated)	35	7.5	5.3	1
Phosphate Buffer	RT	7.4	1.3	1
Phosphate Buffer	RT	11.0	0.29×10^{-4}	1

2.12 Partition Coefficient

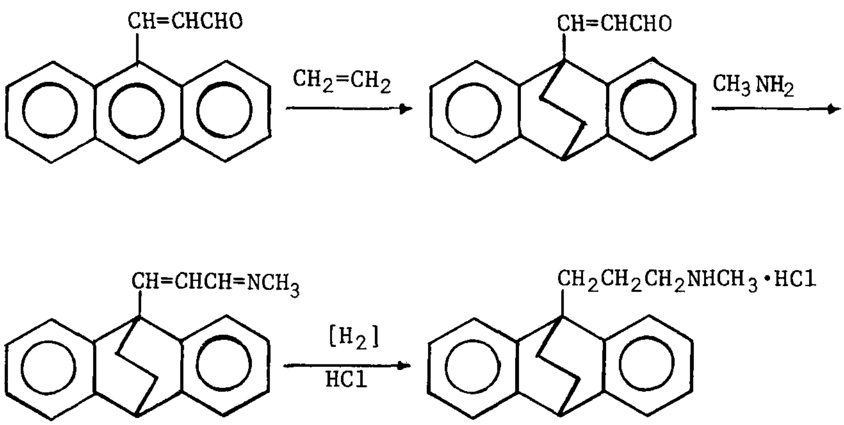
The partition coefficient data shown in Table V were obtained at 25°C as the ratio of the concentration found in the organic phase to that of in the aqueous phase (5, 9).

Table V

Organic Phase	Aqueous Phase	pH	Partition Coefficient
Chloroform	Water	--	0.30
Chloroform	0.1N HCl	--	4.7
Hexane	Water	--	0
n-Octanol	0.1 Hydrochloric Acid	1.1	15.0
n-Octanol	0.1M Glycine Buffer + HCl	3.0	3.51
n-Octanol	1/15M Phosphate Buffer	5.2	1.25
n-Octanol	1/15M Phosphate Buffer	7.4	27.3
n-Octanol	0.1M Glycine buffer + NaOH	9.0	708

3. Synthesis

Maprotiline hydrochloride is synthesized by the following route shown below (10,11):



4. Stability - Degradation

Maprotiline hydrochloride is a very stable compound and resists degradation even in very harsh environments (9, 12). When maprotiline hydrochloride was refluxed for 94 hours in 0.1N HCl and 0.1N NaOH, no degradation product was observed in the sample refluxed in acid, while one trace impurity was detected in the sample refluxed in base. An aqueous solution (0.5%) stored at 100°C for one week showed no decomposition.

Maprotiline hydrochloride is stable after storage for 5 years at room temperature, one year at 50°C, 12 weeks at 40°C/70% relative humidity and 600 f.c. light.

5. Drug Metabolism and Pharmacokinetics

5.1 Metabolism

The lipophilic properties of maprotiline produced by the tetracyclic-propyl portion of the molecule account for the in vivo distribution behavior of this antidepressant. In rodents, following intravenous administration, maprotiline is quickly distributed into organs, with the highest concentrations found in the lungs, adrenal and thyroid glands, and heart. The lowest concentrations are found in the blood. High tissue levels of maprotiline as compared to blood apparently also occur in man, as indicated in a post-mortem study of a suicide from an overdose of maprotiline (13). Following intravenous administration of ^{14}C-labeled maprotiline in man, 57% and 30% of the dose, respectively, were found in urine and feces after 21 days. Maprotiline is excreted in urine predominantly (90%) as metabolites, 75% of which are glucuronides. Principal metabolic transformations involve N-demethylation, deamination, aromatic as well as aliphatic hydroxylations and aromatic methoxy derivative formation (13).

5.2 Methodology

The method most often used in the determination of the pharmacokinetic parameters of maprotiline has been a double radio-isotope derivative technique coupled with thin layer chromatography for specificity (48, 14). A similar double radio-isotope method, with a gas chromatographic

determination for specificity, has been described for the tricyclic nortriptyline (15).

Using an internal standard and a nitrogen detector, maprotiline in plasma has been determined by gas chromatography as the free base (33). When compared to the double radio-isotopic technique, this procedure was found to be comparable in the range of 20 to 150 ng/ml (16). Nitrogen detection by gas chromatography after reaction with acetic anhydride was sensitive to 50 ng/ml for the determination of maprotiline and its N-desmethyl metabolite in blood (30).

Sensitivity to 10 ng of maprotiline in biological samples was achieved using gas chromatography and electron capture detection. Maprotiline was chromatographed as the heptafluorobutyramide after reaction with heptafluorobutyric anhydride (17, 34). Using the same anhydride with either N-desmethylclomipramine or isotope labeled maprotiline as the internal standard, maprotiline can be determined with the combination of gas chromatography and chemical ionization mass spectrometry in biological fluids at concentrations of 0.5 to 150 ng/ml (46).

Reversed phase high perfomance liquid chromatography on a Nucleosil C-18 column with 35% acetonitrile, 65% 0.05 M phosphate buffer (pH 2.7) has also been utilized for the determination of maprotiline in human plasma. Ultraviolet absorbance at 214 nm provided sensitivity to 2 ng/ml of plasma after isolation of maprotiline as the free base (41).

5.3 Pharmacokinetics

The biological half life of maprotiline following a single intravenous dose of 50 mg to 6 healthy volunteers was found to be 43 hours (13). The half life of 40 hours reported in a similar study using normals and a 75 mg intravenous dose, is in excellent agreement (14).

Availability of an oral dose of 50 mg of maprotiline compared to an equal intravenous dose was comparable as determined by AUC measurements (13). Peak levels of maprotiline appear in blood or plasma relatively slowly following oral admin-

istration. Maximum maprotiline levels have been found to occur within about 8-24 hours post administration (13, 18, 19).

The relatively large apparent volume of distribution (13, 14, 19) indicates maprotiline is highly tissue-bound, as the aforementioned post-mortem study indicated (13).

When normal subjects received simultaneously a 50 mg maprotiline tablet and 50 mg of tridueterated maprotiline hydrochloride in an aqueous solution, blood levels for the isotope labeled and unlabeled drug were essentially superimposable. Peak blood level averaged about 50 ng/ml (19).

In five depressive patients receiving seven day incremental doses of 25 mg, 50 mg, and 75 mg, plasma levels of maprotiline were clearly dose related. At the end 7, 14, and 21 days, maprotiline plasma levels were respectively, 18-46 ng/ml, 64-103 ng/ml and 96-155 ng/ml. Although therapeutic activity was observed at all plasma levels (18-155 ng/ml), the incidence of side effects appeared to be related to the 75 mg twice daily dosage regimen (20). Proportional blood levels have also been reported under steady state conditions (about 7 days) for maprotiline doses of 50 mg, 100 mg, and 150 mg (13).

Several reviews and summaries of the pharmacology, pharmacokinetics, toxicity, and efficacy of maprotiline are available (21, 22).

6. Toxicity

The standard intravenous LD_{50} established for maprotiline hydrochloride in male rats is 27.3 mg/kg. Maprotiline hydrochloride is therefore classified as moderately toxic when administered intravenously to rats (23).

7. Methods of Analysis

7.1 Identification

Maprotiline hydrochloride is best identified by: 1) infrared absorption; 2) ultraviolet absorption; 3) a test for chloride.

7.2 Elemental Analysis

The elemental composition determined for a typical sample of maprotiline hydrochloride on a Perkin Elmer Model 240 CHN Analyzer is given below.

Element	Theory, %	Found, %
Carbon	76.53	76.71
Hydrogen	7.71	7.78
Nitrogen	4.46	4.44

7.3 Nonaqueous Titration

Maprotiline hydrochloride may be titrated with acetous perchloric acid in glacial acetic acid containing mercuric acetate. The titration can be carried out potentiometrically using a glass electrode and a calomel electrode containing glacial acetic acid saturated with lithium chloride.

7.4 Phase Solubility Analysis

Phase solubility analysis of maprotiline hydrochloride has been carried out using the following systems:

Solvent: n-propanol
Approximate Solubility: 16.4 mg/g
Temperature: 25°C

7.5 Thin-layer Chromatography

A number of thin-layer chromatographic systems have been developed for the identification and the determination of the drug and compounds related to the drug.

System I

Adsorbent:	Silica Gel G or GF_{254}, 250μ thickness
Mobile Phase:	sec-Butyl Alcohol/Ethyl Acetate/2N Ammonium Hydroxide (120:60:20) saturated with ammonia vapor
Detection:	a) After exposing the plate under hydrogen chloride vapor for 30 minutes, irradiate it with high intensity UV light for 5 minutes. Observe under longwave UV source. b) Iodine vapor

System II

Adsorbent:	Silica Gel G or GF_{254} 250μ thickness
Mobile Phase:	sec-Butanol/Ethyl Acetate/5N Ammonium Hydroxide (14:4:5)
Detection:	After exposing the plate under hydrogen chloride vapor for 30 minutes, irradiate with high intensity UV light for 5 minutes. Observe under longwave UV source.

System III

Adsorbent:	Silica Gel GF_{254}, 250μ thickness
Mobile Phase:	n-Butanol/Acetic Acid/Water (70:10:20)
Detection:	a) Iodine vapor b) After exposing the plate to chlorine vapor for 1-2 minutes, spray with sodium hypochlorite (2%), followed by a second spray of a mixture of 10 ml of KI (1 in 100), 10 ml of soluble starch (3 in 100) and 3 ml of methanol. c) UV 254nm

System IV

Adsorbent:	Silica Gel GF_{254}, 250μ thickness
Mobile Phase:	Chloroform/Methanol (95:5) saturated with ammonia gas
Detection:	Same as System III

System V

Adsorbent:	Silica Gel HF, 250μ thickness
Mobile Phase:	n-Butanol/acetic acid/water (70:10:20)
Detection:	Spray the plate with sodium hypochlorite (2%) in 0.125N sodium hydroxide solution. Dry the plate at 40°C for 1 hour. Spray the plate with a mixture (1:1) of KI (0.83%) and o-tolidine (0.2% in 2% acetic acid).

System VI

Adsorbent:	Silica Gel HF, 250μ thickness
Mobile Phase:	Benzene/Diethylamine (9:1)
Detection:	Same as System V

System VII

Adsorbent:	Silica Gel HF, 250μ thickness
Mobile Phase:	Chloroform/Methanol/Acetic Acid (7:2:1)
Detection:	Same as System V

System VIII

Adsorbent:	Silica Gel GF_{254}, 250μ thickness
Mobile Phase:	Methanol/Ethylacetate/1,2-Dichloroethane/water (3:3:3:1)
Detection:	Same as the detection method b) in System III.

7.6 Gas Chromatography

System I:	The following system has been used to determine the drug substance for routine toxicological analysis (24).
Column:	SE-30 coated fused silica capillary, 3.5 to 15m x 0.25 mm ID
Temperature:	Column programmed from 100°C to 295°C at 5°C/min. Injector & detector temperatures not reported
Carrier:	Helium, linear velocity μ = 45 cm/second at 100°C
Detection:	Flame Ionization
System II	The following system has been used for the determination of the drug substance and other basic drugs in liver (25).

Column:	AR-glass capillaries coated with 0.3% (W/V) SE-52, 15 m x 0.26 mm ID
Temperature:	Injector at 250°C, detector at 300°C, column temperature programmmed by 5°C/min. from 190 to 220°C followed by 15°C/min. to 280°C and then by 8°C/min. to the final temperature of 300°C, at which it was held for 5 min.
Carrier:	Helium, linear velocity 35-40 cm/sec.
Detection:	Nitrogen Phosphorus Detector
<u>System III</u>	The following system has been used for the identification of the drug and its silylated derivatives along with a number of other drug substances and their silylated derivatives (26).
Column:	3% OV-17 on Gas Chrom Q (100-120 mesh) Glass, 2 m x 3 mm ID
Temperature:	Injection port and detector at 300°C, column at 300°C for isothermal run or 120 to 270°C at 10°C/min., hold at 270°C for 16 min. for programmed run.
Carrier:	Nitrogen, 30 ml/min.
Detection:	Flame Ionization
<u>System IV</u>:	The following system has been employed for the determination of the drug substance in plasma, derivatizing it with acetic anhydride (27).
Column:	5% OV-17 on Gas Chrom Q (100-120 mesh), 1.83 m x 2 mm ID glass
Temperature:	Injector and detector at 300°C and column at 255°C
Carrier:	Helium, 30 ml/min.

Detection:	Alkali Flame Ionization
System V:	The following system has been used to determine the drug substance and a number of other neutral and basic drugs in blood (28).
Column:	a) 3% OV-101 on Chromosorb (100-120 mesh), 2 m x 0.6 cm ID glass. b) 3% OV-17 on Chromosorb (100-120 mesh) 2 m x 0.6 cm ID glass.
Temperature:	Injector and detector at 300°C and column from 170 - 290°C programmed at a rate of 4°C/min.
Carrier:	Helium, 30 ml/min.
Detection:	Nitrogen Phosphorous Detector
System VI	The following system has been used for the assay of the drug substance in serum (29).
Column:	1.4% Carbowax 20M plus 1.4% KOH on Gas Chrom Q (60-80 mesh), 1.8 m x 2 mm ID glass
Temperature:	Injection port and detector at 250°C and column at 210°C
Carrier:	Nitrogen, 30 ml/min.
Detection:	Flame Ionization
System VII	The following system has been employed for the assay of the drug substance in biological fluids derivatizing it with acetic anhydride (30).
Column:	3% HI-EFF-8BP on Chromosorb W HP (100-120 mesh), 0.30 m x 3 mm ID.
Temperature:	Injector and detector at 300°C and column at 245°C
Carrier:	Nitrogen, 40 ml/min.

Detector:	Nitrogen Selective Flame Ionization
<u>System VIII</u>	The following system has been employed for the determination of the drug substance in plasma, derivatizing it with heptafluorobutyric anhydride (31).
Column	a) 3% SP-2250 (OV-17) on Supelcoport 80-100 mesh, 2 m x 4 mm ID b) Open tubular column coatded with SE-30, 25 m x 0.37 mm ID
Temperature:	Injector and detector at 300°C and column at 260°C
Carrier:	a) Helium, 50 ml/min. b) Helium, 1.5 ml/min.
Detection:	Nitrogen Selective Detector
<u>System IX</u>	The following system has been used for the determination of the drug substance and other drugs in serum, derivatizing it with trifluoroacetic anhydride (32).
Column:	3% OV-17 on Gas Chrom Q (100-120 mesh), 138 cm x 2 mm ID glass
Temperature:	Injector at 230°C, detector at 270°C and column from 220 - 275°C programmed at 8°C/min.
Carrier:	Nitrogen, 35 ml/min.
Detection:	Nitrogen Phosphorous Detector
<u>System X</u>	The following system has been used to determine the drug substance in plasma (33).
Column	3% OV-17 on gas chrom Q (100-120 mesh), 2 m x 2 mm ID glass
Temperature:	Injector and detector at 300°C and column at 265°C

Carrier:	Not reported
Detection:	Nitrogen Phosphorous Detector
System XI:	The following system has been used for the assay of the drug substance in biological fluids derivatizing with heptafluorobutyric anhydride (34).
Column:	3% JXR (methylsilicone) on Gas Chrom Q, 4' x 3 mm ID
Temperature:	Injection port at 250°C, column at 230°C, detector at 280°C
Carrier:	Nitrogen, 40 ml/min.
Detection:	^{63}Ni Electron Capture
System XII:	The following system has been used to determine the drug substance and its trifluoroacetylated derivative in biological sample (35).
Column:	2% Dexsil 300 on Chromosorb W, AW-DMCS (80-100 mesh), 2 m glass column (column ID not reported)
Temperature:	Injector at 310°C, column at 280°C and detector temperature not reported
Carrier:	Nitrogen, 30 ml/min.
Detection:	Flame Ionization
System XIII	The following system has been used to determine the drug substance in plasma and urine as heptafluorobutyric derivative (36).
Column:	a) 0.75% OV-17 or b) 0.5% XE-60 and 0.25% DC LSX-3-0295 on silanized Chromosorb G (80-100 mesh), 320 cm x 1.8 mm ID glass column
Temperature:	Injector at 255°C, detector at 210°C and column at 245°C

Carrier: Nitrogen at 10 - 15 ml/min.

Detection: ^{3}H Electron Capture

System XIV: The following system has been used to determine the drug substance in blood as heptafluorobutyric derivative (17).

Column: 1.5% OV-225 on Supelcoport packed in a silanized glass column, 1.8 m x 2 mm ID

Temperature: Injector at 250 - 260°C, column at 200 - 230°C and detector at 320 - 330°C.

Carrier: Nitrogen

Detection: ^{63}Ni Electron capture

7.7 High Pressure Liquid Chromatography

System I The following system has been employed for the analysis of the pure drug substance and dosage forms (37).

Column: Zorbax® CN (DuPont), 4.6 mm ID x 250 mm

Mobile Phase: Methanol/0.08M sodium acetate (95:5) with the apparent pH adjusted to 7.5 ~ 8.0

Flow Rate: 2 ml/min.

Detection: Ultraviolet absorption at 272 nm

System II The following system has been employed for the analysis of the pure drug substance and dosage forms (37).

Column: Zorbax® CN (DuPont), 4.6 mm ID x 250 mm

Mobile Phase: Tetrahydrofuran/0.025M sodium acetate (90:10) with the apparent pH adjusted to 7.4 - 8.0

Flow Rate:	1 ml/min.
Temperature:	40°C
Detection:	Ultraviolet absorption at 272 nm
<u>System III</u>	The following system has been employed for the analysis of the drug substance and a number of other basic drugs (38).
Column:	Spherisorb 5 Silica, 4.9 mm ID x 250 mm
Mobile Phase:	a) Methanol/Hexane (85:15) with 0.02 v/v% (1.85 mM) Perchloric Acid b) Same as the above except 0.05 v/v% (4.63 mM) Perchloric Acid c) Same as the above except 0.1 v/v% (9.25 mM) Perchloric Acid
Flow Rate:	2.0 ml/min.
Detection:	Ultraviolet Absorption at 215 nm
<u>System IV</u>	The following system has been employed for the analysis of the drug substance and a number of tricyclic antidepressants (39).
Column:	Hypersil 5 μm ODS, 4.6 mm ID x 70 or 100 mm
Mobile Phase:	a) Acetonitrile/10 mM Sodium Dihydrogen Phosphate (50:50) at pH 2 with 80 mM of sodium lauryl sulfate b) Same as the above except the addition of 5 mM of tetrabutylammonium bromide
Flow Rate:	2.0 ml/min.
Detection:	Ultraviolet Absorption at 254 nm
<u>System V</u>	The following system has been employed for the determination of the drug substance from biological fluids (40).

Column:	μBondapak C_{18}, 3.9 mm ID x 300 mm
Mobile Phase:	Acetonitrile/0.1 M Potassium Phosphate, pH 2.5 (30:70)
Flow Rate:	2 ml/min.
Detection:	Ultraviolet Absorption at 205 nm
<u>System VI</u>	The following system has been used for the assay of the drug substance in human plasma (41).
Column:	Nucleosil C_{18}
Mobile Phase:	Acetonitrile/0.05M Phosphate Buffer, pH 2.7 (35:65)
Flow Rate:	Not indicated
Detection:	Ultraviolet Absorption at 214 nm
<u>System VII</u>	The following system has been used for the qualitative identification of the drug substance and other basic drugs (42).
Column:	Three types of columns have been used. a) 3-7 μm silica b) mercaptopropyl bonded-phase silica c) aliphatic strong cation (n-propylsulphonic acid) bonded-phase silica, 5 mm ID x 250 mm
Mobile Phase:	Methanol/2M Ammonium Hydroxide/1M Ammonium Nitrate (27:2:1)
Flow Rate:	1 ml/min.
Detection:	Ultraviolet Absorption at 254 nm
<u>System VIII</u>	The following system has been used for the assay of biological samples by derivatizing maprotiline with p-nitroazobenzenecarbonyl chloride (43).

Column:	Lichrosorb SI 100 (5μ), 3 mm ID x 100 mm and 3 mm ID x 300 mm connected by two-way switching valve
Mobile Phase:	1.1% t-Butanol, 23% Dichloromethane, 0.05% Morpholine, 0.05% water and make to 100% with Hexane
Flow Rate:	Not indicated
Detection:	Ultraviolet Absorption at 337 nm

<u>System IX</u> The following system has been used for the analysis of the drug substance and a number of tricyclic antidepressants (44)

Column:	Partisil 5 μm, 3.0 mm ID x 150 mm
Mobile Phase:	Dichloromethane/Methanol/Acetate Buffer pH 3.2 (90+10+0.15)
Flow Rate:	1.0 ml/min.
Detection:	Ultraviolet Absorption at 254 nm

7.8 <u>Gas Chromatography - Mass Spectrometry (GC-MS)</u>

<u>System I</u> The following system has been used for the determination of the drug substance and other neutral and basic drugs in blood (28).

Column:	3% SE-30 on Chromosorb (100-120 mesh), 2 m x 0.6 cm ID glass
Temperature:	Injector at 270°C and column from 220 - 270°C programmed at a rate of 10°C./min.
Carrier:	Helium, 30 ml/min.
MS EI and CI Source:	70 eV
CI Reagent Gas:	Methane
Detection:	EI selected ion-monitoring at m/e = 59 and 191 CI selected ion-monitoring at m/e = 278

<u>System II</u>	The following system has been used for the determination of the drug substance and its major metabolite derivatized with trifluoroacetic anhydride using stable isotope-labeled analog as internal standard (45).
Column:	1% OV-17 on Gas Chrom Q (100 - 120 mesh), 1.8 m x 2 mm ID glass
Temperature:	Injector at 240°C, column at 255°C, separator at 250°C, and ion source at 275°C
Carrier:	Helium, 20 ml/min.
MS EI Source:	70 eV
Detection:	EI selected ion-monitoring at m/e = 345
<u>System III</u>	The following system has been used for the determination of the drug substance in biological fluids, derivatizing it with heptafluorobutyric anhydride. Isotope labeled analogs of the drug substance were used as internal standard (46).
Column:	1.5% Poly S-179 on Chromosorb W AW DMCS (80-100 mesh), 1 m x 2 mm ID glass
Temperature:	Injector at 250 - 260°C, column at 240 - 250°C, GC-MS interface at 245 - 270°C and ion source at 245 - 265°C
Carrier:	Not reported
MS CI Source:	40 - 100 eV
CI Reagent Gas:	Methane
Detection:	CI selected ion monitoring at m/e = 474

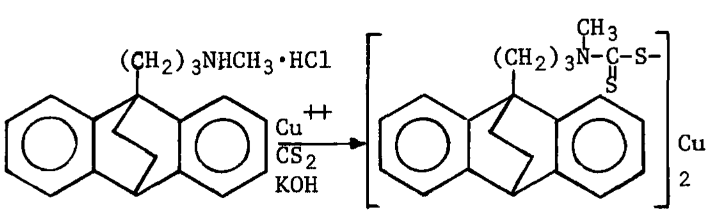
System IV	The following system has been used for the determination of the drug substance and other tricyclic antidepressants in blood plasma, using deuterated internal standards (47)
Column:	3% OV-17 on chromosorb HPW-DMCS, (80-100 mesh), 40 cm x 2 mm ID glass
Temperature:	Injector at 300°C, column at 240°C, separator at 350°C and ion source at 240°C
Carrier:	Helium, 15 ml/min.
MS CI Source:	70 eV
CI Reagent Gas:	Methanol Vapor
Detection:	CI selected ion-monitoring at m/e = 278/286 [$(M+1)^+$ Ludiomil/$(M+1)^+$ deuterated Ludiomil]
System V	The following system has been used for the determination of the drug substance and its trifluoroacetylated derivative in biological sample (24).
Column:	1% OV-17, 2 m glass (solid support and column ID not reported)
Temperature:	Column at 150-320°C, programmed at a rate of 15°C/min. Temperature for injector and ion source not reported.
Carrier:	Not reported
MS EI Source:	70 eV
Detection:	EI selected ion-monitoring at m/e = 345

7.9 Double Radio-Isotope Derivative Method

The double radio-isotope derivative technique for the assay of the drug substance in biological material has been employed by Riess (48). A biological sample spiked with a definite amount of ^{14}C-labeled maprotiline standard was extracted with heptane containing 1% amyl alcohol. The extracted sample (^{14}C-standard plus unknown) was derivatized with ^{3}H-acetic anhydride. After the addition of an amount of unlabeled acetyl derivative of maprotiline as carrier, the sample was purified by two-dimensional thin-layer chromatography. The purified sample was analyzed for both ^{3}H activity and ^{14}C activity by liquid scintillation counting with the discriminator-ratio method. Appropriately prepared standard samples allow calculation of the overall individual yield of the ^{14}C-internal standard in each sample. By means of the ^{14}C-yield found, the recovered ^{3}H activity value can be corrected to the equivalent of a theoretical 100% yield. The resultant ^{3}H activity value can be converted to the concentration units by the 100% ^{3}H activity value resulting from an appropriate number of tests samples which have been treated in exactly the same way as the unknown and contain a definite, known amount of trial compound.

7.10 Colorimetric Assay

Maprotiline hydrochloride can be assayed by the dithiocarbamic acid copper complex method. Maprotiline, a secondary amine, undergoes the following reaction with carbon disulfide and cupric ion in an alkaline medium.



The absorbance of the colored complex is measured at 437 nm. The method is specific to the secondary aliphatic amine.

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PENICILLIN G, POTASSIUM

(POTASSIUM BENZYLPENICILLIN)

JOEL KIRSCHBAUM

1. Introduction

1.1 History

In 1928, Alexander Fleming at St. Mary's Hospital in London noticed the partial lysis of colonies of staphylococci on a plate that had been contaminated by *Penicillium notation*. Previous observations of the antagonism of the growth of bacteria by fungi of the genus *Penicillium* had been made between 1870 and 1895, however Fleming further found that this fungus, when cultured, gave a "mould broth filtrate" that was relatively non-toxic, possessed selective antibacterial activity and could be used as a local antiseptic. He believed that "the trouble of making it seemed not worthwhile". For the next few years, little new information on the properties and purification of this substance was obtained. As the second world war approached, sulfa drugs were the only known useful way to treat infections chemically. Exploratory research by E.B. Chain, Howard Florey, N.G. Heatley and E.P. Abraham, all at Oxford University, yielded small quantities of impure penicillin.

Because of the bombings of Britain, Florey and Heatley went to the United States to obtain aid, after a close escape in Lisbon from being arrested for swimming topless. Work in the United States with deep fermentation (rather than surface growth) and corn steep liquor as a growth promoter, with the efforts of personnel at Squibb, Pfizer, Merck and several other organizations, resulted in the eventual production of sufficient antibiotic for both battle casualties, and, subsequently, civilians. The Germans concentrated on sulfa drugs, although there was sufficient fermentation capacity, considering beer production.

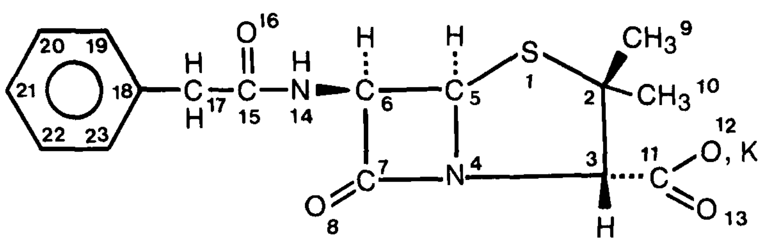
Penicillin G was first crystallized at Squibb (as a sodium salt) by MacPhillamy, Wintersteiner and Alicino, where the existance of sulfur in the molecule was first noted by Alicino due to its characteristic odor. The β-lactam structure was supported by R.B. Woodward, especially since many other possible reactive groups could be eliminated by such evidence as potentiometric titrations that indicated the absence of a basic functionality.

The structure of benzylpenicillin was established by Dorothy Hodgkin and Barbara Lois in May, 1945, using three-dimensional x-ray crystallography. Thus, it was shown that penicillin contained a previously unknown ring system. The β-lactam ring is the source of antibacterial activity.

Fleming, Chain and Florey shared the Nobel prize in 1945. Fleming received popular honors for the discovery of penicillin. For example, in the fishing village of Gijon, Spain, the aggrandized photograph of Sir Alexander was paraded about at the yearly fiesta with the caption, "To the Holy Virgin we pray: for us, many Sardines; for the wizard who gave us penicillin, Glory".

1.2 Names, Formula and Molecular Weight

Penicillin G Potassium is the United States adopted name (10). The preferred chemical names are (1) 4-Thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-,monopotassium salt, [2S-(2α,5α, 6β)]-; and (2) Monopotassium (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate. The chemical abstracts systematic number for potassium penicillin G is CAS-113-98-4; CAS-61-33-6 is for penicillin G itself. Below is the structure, $C_{16}H_{17}KN_2O_4S$, with molecular weight of 372.48 daltons (11). Other names include benzylpenicillin potassium,



potassium benzylpencillinate, benzylpenicillinic acid potassium salt, Cilloral, Cosmopen, Cristapen, Crystapen, Eskacillin, Forpen, Hipercilina, Hydsorb, Hylenta, Liquapen, M-Cillin, Megacillin tablets, Monopen, Notaral, Penisem, Pentid, Scotil, SK-Penicillin G, Sugracillin and Tabilin. Benzyl-

penicillin has been formulated in tablets (12), ointments (13,14), lotions (15) and intravenous solution (16). It has been added to drinking water (17) and sutures (18), and conjugated to lysine to minimize immune reactions (19).

1.3 Appearance, Color, Odor and Precautions

Potassium benzylpenicillin is a white, free-flowing crystalline powder with a faint pungent odor characteristic of penicillins. The compound itself should not be mixed with acids, alcohol, bases, ephredrine, glycerol, iodine and iodides, naphthalene oils, oxidizing agents, resorcinol, salts of heavy metals, vitamin B_1 and zinc oxide (*cf*. Section on Stability).

The significant adverse reaction to benzylpenicillin is the induction of an allergic reaction as servere as anaphylactic shock leading to death. Numerous studies (20) have shown that high and low molecular weight compounds were responsible (21); including penicillin itself, penicilloyl derivatives (such as penicilloyl-lysine and penicilloyl-albumin), oligomers and degradation products (such as penicilloic acid, benzylpenicilloate, monopenicilloyl amides, *D*-penicillamine and *D*-penicillamine-*L*-cysteine) (22, 23) which can elicit an immediate reaction in patients allergic to penicillin. As expected, different commerical preparations of benzylpenicillin vary in their ability to evoke allergic reactions (24).

1.4 Biosynthesis, Synthesis, and Commercial Production

1.41 Biosynthesis

Beta-lactam compounds are formed in nature as secondary metabolites of both eukaryotic and prokaryotic organisms. The penicillins, cephalosporins and 7α-methoxy-cephalosporins are products of biosynthetic pathways with many identical steps (26). The reaction of an activated form of *L*-α-aminoadipic acid (II, Fig. 1) with *L*-cysteine to form a common precursor

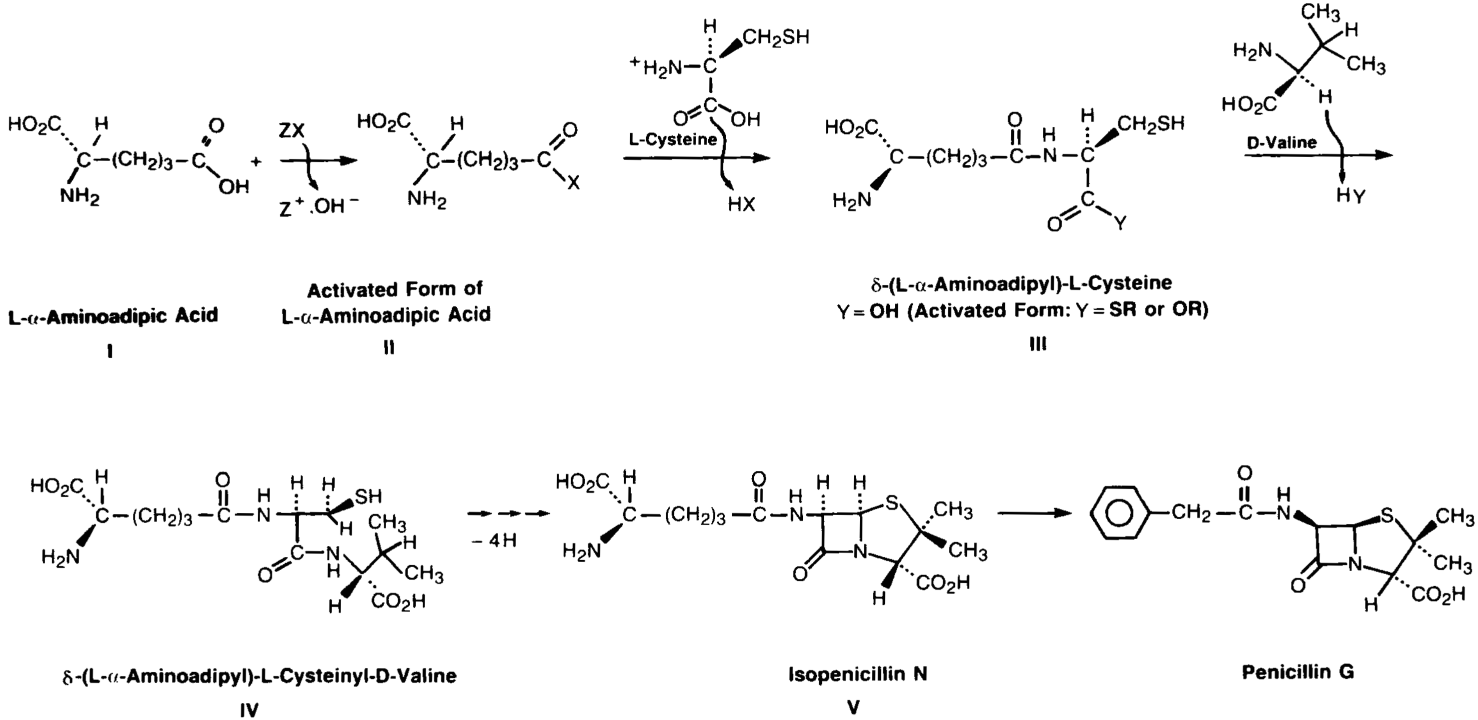


Figure 1. Biosynthesis of Penicillin G.

δ-(*L*-α-aminoadipyl)-*L*-cysteinyl-*D*-valine (IV, 27). Cyclization proceeds via an enzyme, cyclase, dependent on ferrous ion and ascorbate oxidation to yield isopenicillin N (28). Isopenicillin N is converted to penicillin N through the action of an epimerase which converts the *L*- to a *D*- form, leading to both penicillins and cephalosporins (29), Fig. 3. Interconversions between penicillins are possible using enzymes (30) in the reactions shown below: 6-APA is 6-aminopenicillanic acid,

[^{35}S]Penicillin V + 6-APA $\rightleftharpoons$ Pen V + [^{35}S] 6-APA
Penicillin G + [^{35}S]6-APA $\rightleftharpoons$ [^{35}S]Penicillin G + 6-APA

[^{35}S]Penicillin V+ Penicillin G $\rightleftharpoons$ Penicillin V+[^{35}S] Penicillin G

A chemical synthesis similar to the biosynthetic sequence involving the carbon-hydrogen bond-breaking steps of IV at the cysteinyl-β- and valinyl-β-positions has been devised (31).

1.42 Synthesis

The first successful synthesis of penicillin (25) is shown in Figure 2 and, when modified, can be used for related penicillins.

1.43 Commercial Production

Originally, penicillin G was so rare that it was repurified from the urine of patients. Now, with large-scale production (32), the cost is 20¢ (U.S.) a day based on a four tablet-a-day regimen and a 100% profit margin. Penicillin G is also used to synthesize other β-lactam antibiotics. Radioactive penicillin G has been produced from *L*[1-^{14}C]-valine by mycelia of *Penicillium chrysogenum* immobilized in an alginate gel (35).

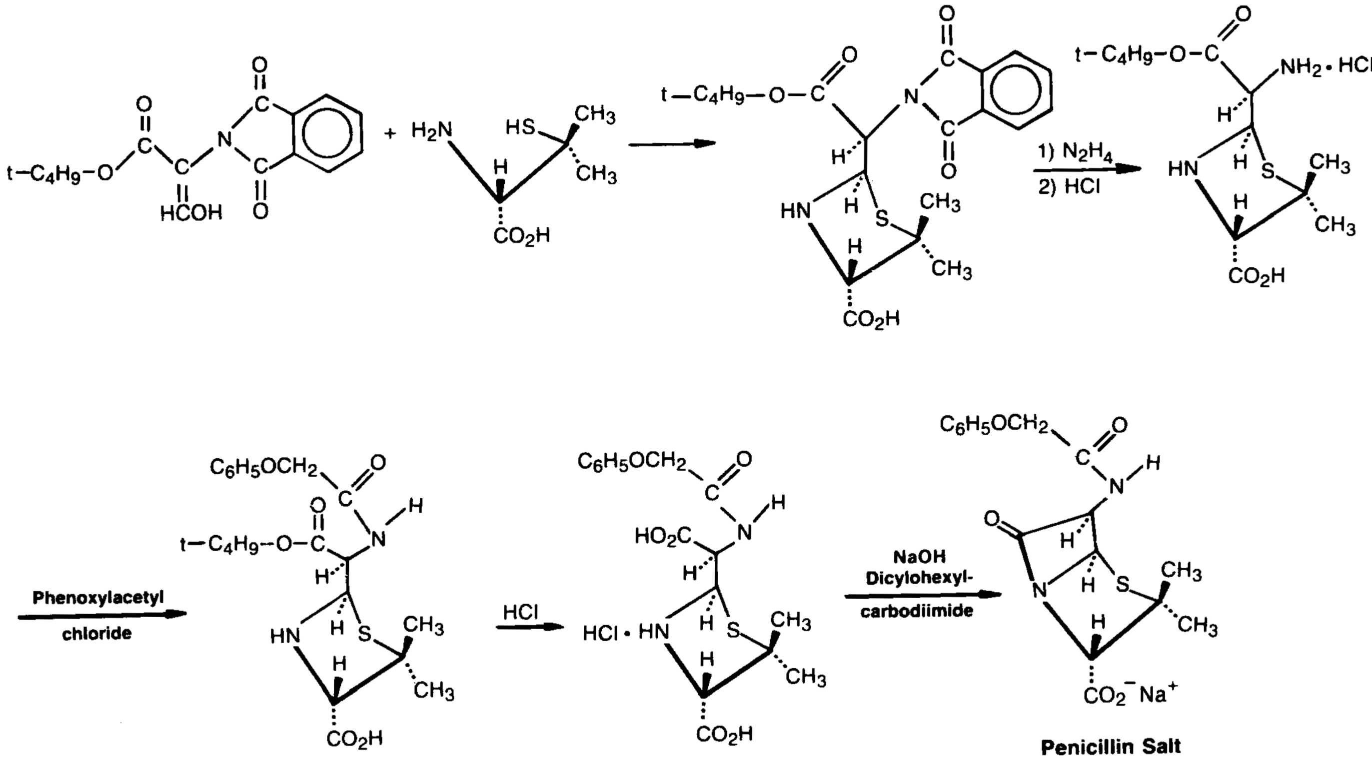


Figure 2. Synthesis of a Penicillin.

1.5 Reactions, Stability, Degradation and Polymerization

1.51 Reactions,

Potassium penicillin G can be converted to the sodium salt using cation exchange resin (36,37) and to other salts (38,39).

Antimicrobial activity is lost by hydrolysis of the β-lactam ring, which is catalyzed by various β-lactamases induced or naturally occurring *in vivo* (40-42); *cf.* stability section *v.i.* for studies of hydrolysis *in vitro*. Other reactions of the β-lactam group include aminolysis (43,44) and amino group conjugation (45,46). Benzylpenicillin has been converted to penicillanic acids (47) and penicillamine (48,49) enzymatically, to 5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid (50) and, via an oxygen bridge at C-6, to a dimer (51).

Removal of the acyl side chain, either chemically (52,53) or enzymatically (54-56) leaves an active nucleus, 6-aminopenicillanic acid, that can be reacted with other groups to create various semi-synthetic penicillins. The side chain can undergo various reactions (57-59).

The carboxylic acid moiety may readily be converted to esters (60,61). The β-lactam can be reduced (62) to an amino alcohol and the sulfur oxidized (63-65). Penicillin sulfoxide can be converted to cephalosporins (66) as well as the protected ester (67). To investigate monocyclic β-lactams, penicillin was desulfurized with Raney nickel (68). Benzyl-penicillin reacts with metals like mercury (II) acetate to form the azetidinone (69) via 1,5-bond scission (70), *cf.* the stability section above for less lucid metallic effects.

A penicillin antigen can be formed by the conversion of benzylpencillin to *D*-benzyl-

penicillenic acid followed by coupling with β-alanine (71). Other artificial antigens were created via coupling with lysine (72), polyacrylamide (73) and diaminocarboxymethyl-levan (74).

Noncovalent bonding of benzylpenicillin was studied in microorganisms (75), to serum albumin by NMR (76) with the binding sites localized (77), to serum (78) and to tissues (79) as well as bacterial proteins (*cf.* section on mode of action).

1.52 Stability and Degradation

The kinetics and stability of penicillin G have been studied extensively. Even the effect of high concentrations of potassium benzylpenicillin was investigated, since it was found that micellar penicillin G is 2.5 times as stable as the non-micellar solution under the conditions of constant pH and ionic strength (80).

Effect of pH

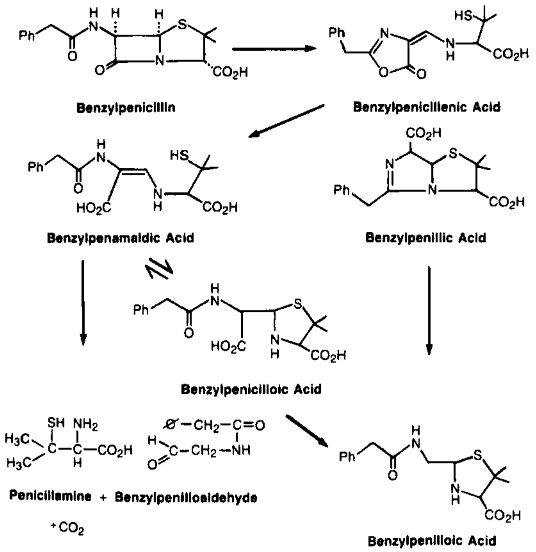
The stability of a commercial preparation of potassium penicillin G in water, 0.1 M NaOH and 0.1 M HCl was studied using ultraviolet spectrophotometry (81).

Solvent and Concentration	Wavelength (nm)		Time (hr) 0	1	3	5	24
Water 2.7×10^{-3} M	262	Molar Abs.	164	163	163	164	164
	256		251	250	250	251	250
0.1 M HCl 1.4×10^{-4} M	273	Molar Abs.	1435	1406	1385	1392	1343
0.1 M NaOH 2.9×10^{-4} M	263*	Molar Abs.	160	160	162	164	178
	257		223	223	222	223	234
	251		218	217	215	214	219
	246*		211	209	205	203	200

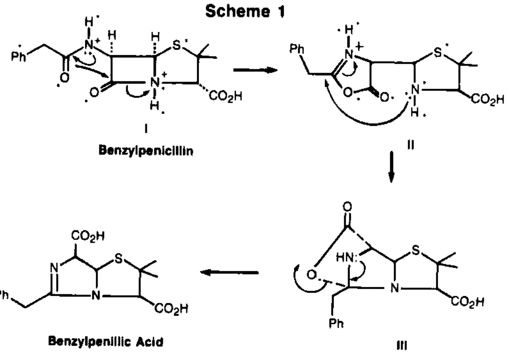
*Shoulder, Instrument: Perkin-Elmer Model 320

From these data, and especially from the studies discussed below, benzylpenicillin is shown to be most stable at neutral pH. Low temperature also enhances stability. The activation energies were reported to be 17.6, 21.0 and 22.7 kcal/mole at pH 1.2, 4.54 and 9.57, respectively.

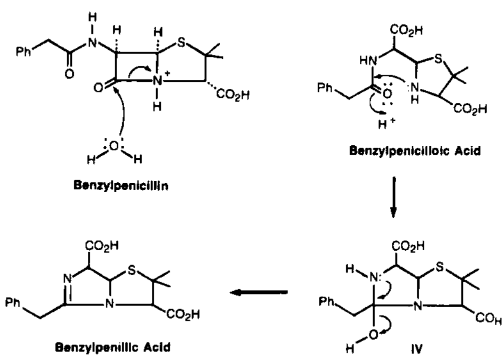
Several pathways have recently been proposed for the degradation of benzylpenicillin (I) at low pH. Evidence for scheme 1 is based on kinetics and the existance of penicillamine as demonstrated by HPLC and differential pulse polarography (82). An alternative route to benzylpenillic acid involves intermediate II depicted in scheme 2 (83). Evidence is based on (a), the increased stability of benzylpenicillins by the incorporation of electron-withdrawing substituents in the α-position of the amide side-chain and (b), that benzylpenicillinic acid in ethanol yields benzylpenillic acid in 25% yield. Scheme 3, showing the conversion of benzylpenicilloic acid to benzylpenillic acid via a carbinolamine intermediate (IV), is based on HPLC data (84) showing such a degradation at pH 2.5 of benzylpenicilloic acid to benzylpenillic acid. Another alternative scheme (85), not shown here, lacks such a pathway for the formation of benzylpenillic acid from benzylpenicilloic acid, although the kinetics are supported by NMR.



Scheme 1



Scheme 2



Scheme 3

Figure 3. Possible Pathways for the Degradation of Benzylpenicillin

Below are tabulated other selected references describing the stability of benzylpenicillin under various conditions.

Stability Condition	Comments	Reference
Bulk (Pure) Penicillin		
pH, ionic strength, temp.	Study side-chains	86
Acid	Study mechanism	87
Acid	Study kinetics	88
pH	Kinetics	89,90
Ionic strength, temp.	Effect ionic strength	91
Acids, bases, temp.	Determine best stability	92
Buffers	Effect phosphate buffers	93
Intravenous Solutions		
Dextrose & Sucrose, Inactivated	Used HPLC	94
In 10 Solutions	Hydroxylamine assay	95
In 7 Solutions	Retained activity	96
In 4 Solutions	Student experiments	97
In 3 Solutions	Study effect time and temperature	98,99
In 2 Solutions	6-8 hr stability	100
In dextrose + 10 drugs	Predict stability	101
In minibags	Effect temp.	102
In dextrose	Effect pH	103
Hyperalimentation	Effect temp. and nutrients	104-107
Y-Injection	Study incompatibilities	108-110
Unit Doses	Effect time	111
Other Formulations		
Sensitivity discs	Effect temperature	112
Topical	Metals degrade	113
Suppositories	Effect excipients	114

Temperature

Heat	Study kinetics solids	115
Heat	Effect water	116
Heat	Predict stability	117
Freezing	Effect dextrose, NaCl with time	118
Freezing	Effect mode freezing	119
Freezing	Effect various thaws	120
Freezing	Study phase transitions	121

Chemicals (also see Intravenous Solutions)

Alcohols	Little effect	122
Amikacin	Little effect	123
Ascorbic Acid	Little effect	124
Broth	High productivity = low stability	125
Broth	Production depends on syn. rate and pencillin stability	126
Magnesium Sulfate	Depends on pH	127
Metals	Penicillin → penicilloic acid	128
Metals	In culture medium	129
Preservatives	Crystallize	130
Silica	Surface acidity causes degradation	131
Sucrose	1:1 Molar complex	132
Sucrose	Study kinetics pH 6-10	133
Surfactants	Stability depends on ionic type	134
Surfactants	Micelles-penicillin complex	135
Tobramycin	[β-methyl-^{3}H][^{14}C]benzylpenicillin Hydroxylamine prevents loss	136
Tromethamine	Aminolysis	137
Water	Causes degradation	138

Radiation

Solid State	Decomposition & dimerization	139,140
UV	Bacterial inhibited none	141

The deuterium isotope effect with pH (pH 4-10) was studied (142) and demonstrated a water-catalyzed rearrangement of penicillin G to benzylpenicillenic acid (scheme 1). A microcomputer was used to help predict the stability of penicillin formulations (143). Degradation products of double-labeled [β-methyl-^{3}H] [^{14}C]benzylpenicillin were separated (144) using a cation-exchange column (*cf.* thin-layer and high-performance liquid chromatography section for additional references) after storage under various conditions.

Of particular importance in the treatment of infection with penicillin G is the complex interplay possible with certain other drugs (145).

1.53 Polymerization

Polymers of such β-lactam antibiotics as benzylpenicillin elicit a passive cutaneous anaphylactic reaction in sensitized animals. Various methods have been used to clarify the antigenic determinants relative to the allergic reactions evoked by the administration of penicillin G containing polymers and β-lactam reaction products with proteins.

Dextran gels Sephadex G-10 and G-25 (size-exclusion chromatography) was used to separate various reaction products of penicillin G (146).

Aged, 25% solutions of penicillin G were chromatographed on G2000SW and G-25 columns (147). Polymer contents increased with duration of storage. These major fractions were found after 14 days storage in the dark, following chromatography on Sephadex G-25. Two fractions consisted of equimolar phenylacetyl and thiazolidine moities and had C:N:S ratios similar to that of penicillin G. These polymers appeared to be trimers and decamers. The third fraction was mainly N-formylpenicillamine, benzylpenilloic acid, benzylpenicilloic acid and benzylpenillic acid, using ^{1}H NMR and IR spectra, and thin-layer chromatography (148). These appear to be

products resulting from the opening of the β-lactam ring, as was previously suggested in a prior study of penicillin polymers (149) using gel exclusion separations and IR, UV and NMR techniques.

1.6 Mode of Antibacterial Action

Penicillin enabled the cure of many common, often serious infections such as meningococcal meningitis, pneumococcal pneumonia, group A streptococcal pharyngitis as well as most staphlococcal and many skin infections. Prior to the advent of penicillin, bacterial endocarditis was almost uniformly fatal, with less than a 1% rate of spontaneous healing; however, treatment with penicillin resulted in cures. Unfortunately, resistance to penicillin G emerged rapidly. However, synthetic modifications to the β-lactam structure preserved many of the therapeutic gains and, in some compounds, produced new advantages.

The targets of β-lactam antibiotics are the enzymes involved in synthesizing the cell wall surrounding most bacteria. The cell wall of gram-positive bacteria consists of 50 to 100 molecular layers of peptidoglycan, which is a long linear polysaccharide chain of alternating N-acetyl-glucosamine and N-acetylmuramic acid extending in one direction, and cross-linked by short peptides in a second dimension. These peptidoglycan strands have a dipeptide terminus, acyl-*D*-alanyl-*D*-alanine, that may assume a similar conformation to penicillin (150). The peptide bond cleaved during transpeptidation (which leads to incorporation and insolubilization into the peptidoglycan cell wall) would be in the same position as the highly reactive CO-NH bond in the β-lactam ring of penicillin. Normally, the transpeptidase is thought to react with its R-*D*-alanyl-*D*-alanine substrate to form an acyl-enzyme intermediate as the terminal *D*-alanine is simultaneously eliminated (151). Cross-links are formed as a free amino group from an adjacent glycan strand reacts with an amino acceptor to yield the transpeptidase product and concurrently regenerate the enzyme. Penicillin inhibits this reaction, presumably by acting as a structural analog of the substrate, via reaction of the β-lactam with the active site, to form an inactive penicilloyl-enzyme

(Fig. 4). Inhibition of peptide cross-linking results in the production of linear, soluble, peptide-substituted glycan chains that are not linked to the insoluble cell wall, but instead are excreted as peptideglycan. This often results in the lytic death of the microorganism. (Except when beta-lactamases enable cell wall synthesis to proceed and thus account for penicillin resistance). Evidence for the peptidoglycan hypothesis is 1) the existance of proteins that covalently bind penicillins (152), 2) the reversal by hydroxylamine of penicillin binding to membranes to give a penicilloylhydroxamate with the concomitant restoration of cell wall biosynthetic activity, and 3) the binding of both a penicilloyl moiety and an acyl moiety (derived from substrate) to the same amine acid residue at the active site of sensitive enzymes in stoichiometric quantities (153,154). Additional evidence is from the evolutionary relationship between β-lactamases and penicillin-sensitive enzymes of cell wall biosynthesis, as shown by sequence homology and similar secondary structure. When 48 strains of the soil bacterium *Rhizobium japonicum* were screened for their responses to several widely used antibiotics, 25% were found to be resistant to penicillin G, tetracycline, neomycin, chloramphenicol and streptomycin. The occurence of multiple drug resistance in a soil bacterium that is not a vertebrate pathogen suggested that the therapeutic use of antibiotics may not be required to develop multiple drug resistance (155). Resistance appears to depend (156) on β-lactamase activity and penicillin-binding protein alterations. Evidence against this theory is the lack of analogy to the dipeptide of portions of both rings of penicillin and the slight activity of some penicillins and cephalosporins with structural analogies to acyl-*D*-alanyl-*D*-alanine. Solid state ^{15}N-NMR studies using *L*-[ε-^{15}N]lysine and benzylpenicillin indicated that disruption of the mechanism for control of peptidoglycan synthesis probably is a contributing factor in the death of bacteria (157).

1.7 Non-Microbiological Effects

In addition to the antimicrobial activity of potassium benzylpenicillin, in rabbits penicillin

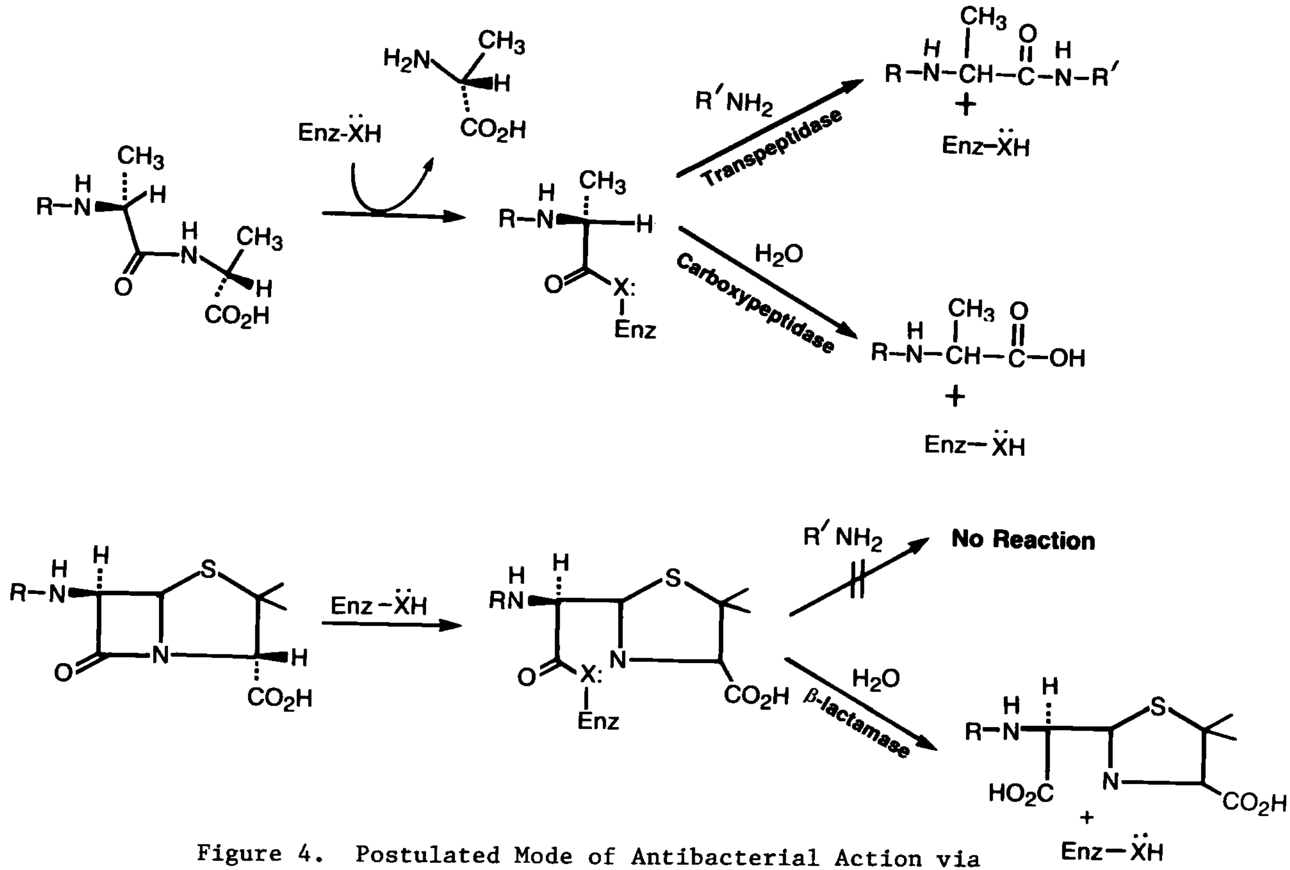


Figure 4. Postulated Mode of Antibacterial Action via Inhibition of Cell Wall Synthesis.

administered intramuscularly increased cytochrome C oxidase activity and decreased the concentrations of free SH and -S-S- (158). Red blood cells in healthy children showed higher potassium concentrations after the addition of therapeutic concentrations of benzylpenicillin (159). Sodium excretion in urine was significantly higher (160). A diet containing 4% potassium penicillin G resulted in the inhibition of collagen biosynthesis and cross-linking (161). Suppression of intestinal bacteria by benzylpenicillin resulted in changes in the concentrations of amino acids in tissues (162). Lipid metabolism in microorganisms was also affected by penicillin G (163). Symptoms of Lyme arthritis (spirochete infection-caused) are relieved by penicillin (164).

1.8 Pharmacokinetics and Metabolism

In man, 15-30% of the total oral dose is absorbed, due, to a great extent, to the low stability of penicillin G in stomach acid. Lower percentages are absorbed if benzylpenicillin is administered during a meal (165-167). Disappearance of drug from the gut lumen depends on the intrinsic absorption rate and the non-enzymatic rate of degradation (168). Approximately 60% of the antibiotic is bound to serum proteins. Peak serum concentrations after a 500 mg oral dose (fasting) are 1.5-2.7 μg/mL of total drug and 0.6-1 μg/mL of free drug. This concentration can inhibit streptococci, staphylococci and pneumococci. Infusion of 500 mg/kg/hour gave 16 μg/mL total drug and 5.6 μg/mL free drug (165).

For potassium penicillin G, the half-life is 5 min. (Orally active penicillins typically give values of 180 and 1160 min.), with the reaction following pseudo-first order kinetics (169). The effect on bacteria of this rapid decline in concentrations was studied (170).

Distribution of injected potassium benzylpenicillin depends on the dose (171). Lower concentrations are found in cerebrospinal fluid than in serum (172), due to the blood brain barrier. High doses of penicillin provide a minimum inhibitory concentration. Bone shows no detectable penicillin even when serum concentrations are as high as 9 μg/mL

(173). Alveolar macrophages also show restricted entry (174). Penicillin is principally excreted in the urine (175), usually in unmetabolized form (10-30%). Biliary excretion also occurs (176,177) but is almost negligible. Biotransformation results mainly in inactive penicilloic acids. Pharmacokinetics were also studied in turkeys (178), horses (179), swine (180), and rabbits (181). A synthetic, oxygen carrying, resuscitation fluid (perfluorocarbon emulsion Fluosol DA 20%) showed no significant difference in pharmacokinetic parameters (182).

2. Physical Properties

2.01 Single Crystal X-Ray Diffraction

The original three-dimensional structure was obtained by Dorothy Crowfoot Hodgkin and coworkers (183), which served to establish the structure of the β-lactam ring and the location of the sulfur atom. However, due to the large fluctuations in the background intensity of electron density and the abnormal interatomic distances, additional refinements were required to define the benzene ring (184). Fig. 5 depicts the structure obtained using Mo-K_{α} radiation measuring 1875 reflections using 1° $\min^{-1}$ Θ-2Θ scans (185). The bond distances and angles are tabulated in Table 1. Crystal data are as follows: $P2_1 2_1 2_1$; U/A°$_3$ = 1,770.9, D_m/g cm^{-3} = 1.359; a = 9.303 (2), b = 6.342 (3) and c = 30.015 (a), and Z = 4.

Of greatest interest is that four of the five atoms of the thiazolidine ring tend to be co-planar, the conformation of the thiazolidine ring depends on the local environment and the interior angle of the S atom is 94.7°. The potassium ion is co-ordinated by seven oxygen atoms. Biological activity correlates with the conformation of the thiazolidine ring (186). The three-dimensional structure was compared to the spatial characteristics of glycyglycine, a model of the *D*-alanyl-*D*-alanine terminus of the precursors of bacterial cell-wall peptidoglycan cross-links (187) and to acyl-*D*-alanyl-*D*-alanine (188) itself. Structures were obtained for the related 6-aminopenicillanic acid (189), benzylpenicillin 1'-diethyl carbonate ester (190) and phenoxy- methyl-anhydropenicillin (191), whose stereochemical structure was related to other penicillins.

These data may be used to calculate a conformational energy map depicting the conformations possible for the β-lactam-thiazolidine ring system with respect to the side-chain (192).

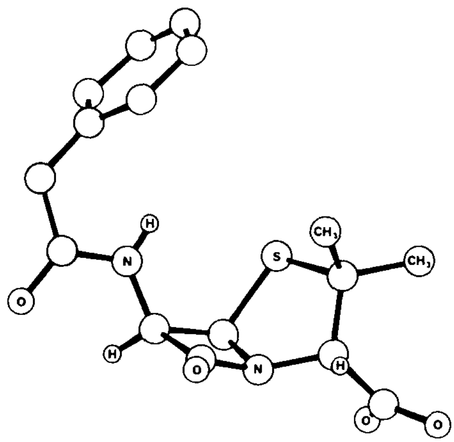


Figure 5. Three-Dimensional, X-Ray Derived, Structure of Potassium Penicillin G.

Table 1. Bond distances and angles

Distances	(Å)
S(1)-C(2)	1.847(10)
S(1)-C(5)	1.818(9)
C(2)-C(3)	1.57(1)
C(3)-N(4)	1.46(1)
N(4)-C(5)	1.45(1)
N(4)-C(7)	1.38(1)
C(5)-C(6)	1.56(2)
C(6)-C(7)	1.52
C(7)-O(8)	1.21
C(2)-C(9)	1.52
C(2)-C(10)	1.53
C(3)-C(11)	1.55
C(11)-O(12)	1.23
C(11)-O(13)	1.25
C(6)-N(14)	1.43
N(14)-C(15)	1.34
C(15)-O(16)	1.22
C(15)-C(17)	1.51
C(17)-C(18)	1.45
C(18)-C(23)	1.33
C(23)-C(22)	1.37
C(22)-C(21)	1.28
C(21)-C(20)	1.32
C(20)-C(19)	1.41

Angles (°)	Degrees
C(2)-S(1)-C(5)	95.2(4)
S(1)-C(2)-C(3)	106.2(6)
C(9)-C(2)-C(10)	110(1)
C(2)-C(3)-N(4)	105.2(7)
C(2)-C(3)-C(11)	113.9(7)
N(4)-C(3)-C(11)	111.3(7)
C(3)-N(4)-C(5)	119.4(7)
C(3)-N(4)-C(7)	125(1)
C(5)-N(4)-C(7)	94.1(8)
S(1)-C(5)-C(7)	105.1(6)
S(1)-C(5)-C(6)	119.7(6)
N(4)-C(5)-C(6)	88.3(7)
C(5)-C(6)-C(7)	84.1(7)
C(5)-C(6)-N(14)	117.3(7)
C(7)-C(6)-N(14)	115(1)
N(4)-C(7)-C(6)	93(1)
N(4)-C(7)-O(8)	131(1)
C(6)-C(7)-O(8)	137(1)
C(3)-C(11)-O(12)	117(1)
C(3)-C(11)-O(13)	116(1)
O(12)-C(11)-O(13)	127(1)
C(6)-N(14)-C(15)	121.1(1)
N(14)-C(15)-C(17)	117(1)
C(17)-C(15)-O(16)	122(1)
C(14)-C(15)-O(16)	121(1)
C(15)-C(17)-C(18)	118(1)
C(17)-C(18)-C(23)	124(1)
C(17)-C(18)-C(19)	122(1)
C(18)-C(23)-C(22)	128(2)
C(23)-C(22)-C(21)	115(2)
C(20)-C(21)-C(22)	125(2)
C(21)-C(20)-C(19)	118(2)
C(20)-C(19)-C(18)	120(2)
C(23)-C(18)-C(19)	114(1)

2.02 X-Ray Powder Diffraction

To observe x-ray diffraction patterns, a Philips APD 3720 Automated Powder Diffractometer unit emitting CrK_{α} radiation at 2.2897°A was used (193). The upper portion of Figure 6 shows the powder x-ray diffraction pattern for potassium penicillin G, the lower portion a stick plot of the crystalline peaks, using the same data, where the y-axis (height) represents intensity and the x-axis represents the diffraction angle at the peak maximum in degrees 2Θ. Below are the data for the most intense peaks.

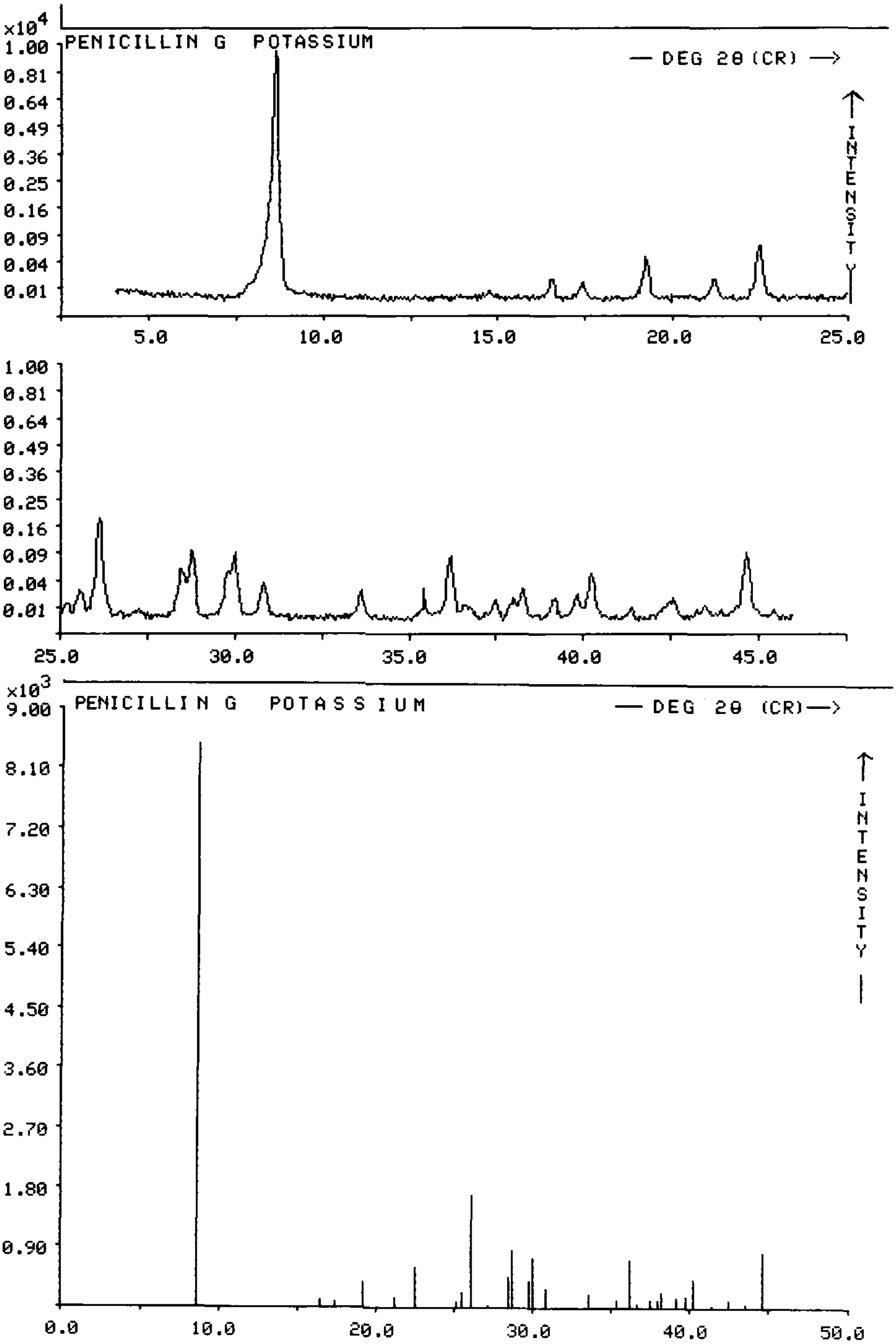
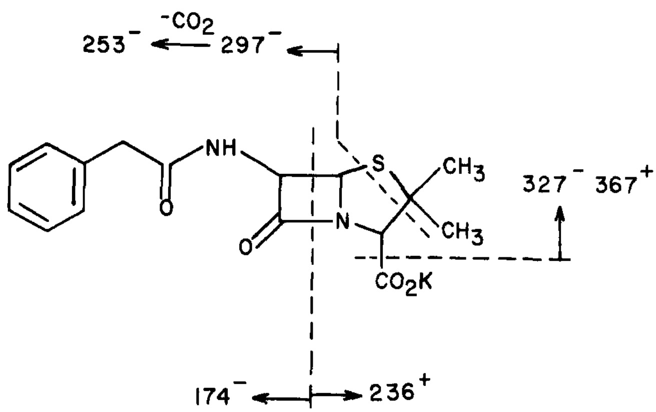


Figure 6. Powder X-Ray Diffraction Patterns; Upper Portions, Conventional Plot; Lower Portion, Stick Plot.

Peak Angle	D Spacing (Å)	I/I Max (%)
8.6250	15.2248	100.0
26.1050	5.0692	20.9
28.7600	4.6098	10.08
44.6600	3.0132	9.74
29.9850	4.4255	8.95
36.1800	3.6870	8.63
28.4400	4.6606	5.54
40.2250	3.3294	5.09
29.7400	4.4611	4.81
19.2250	6.8561	4.39

2.03 Mass Spectrometry

Positive and negative mass spectra (Fig. 7) of potassium benzylpenicillin were obtained (194) from a thioglycerol matrix using a 8 KeV Xe fast atom beam. The resulting secondary ions were mass analyzed using a VG-ZAB-2F mass spectrometer. Fig. 7 shows fast atom bombardment MS/MS spectra of the potassiated parent (top) and of the deprotonated molecule (bottom). The suggested fragmentation is shown below, which is general for many penicillins and highly diagnostic.



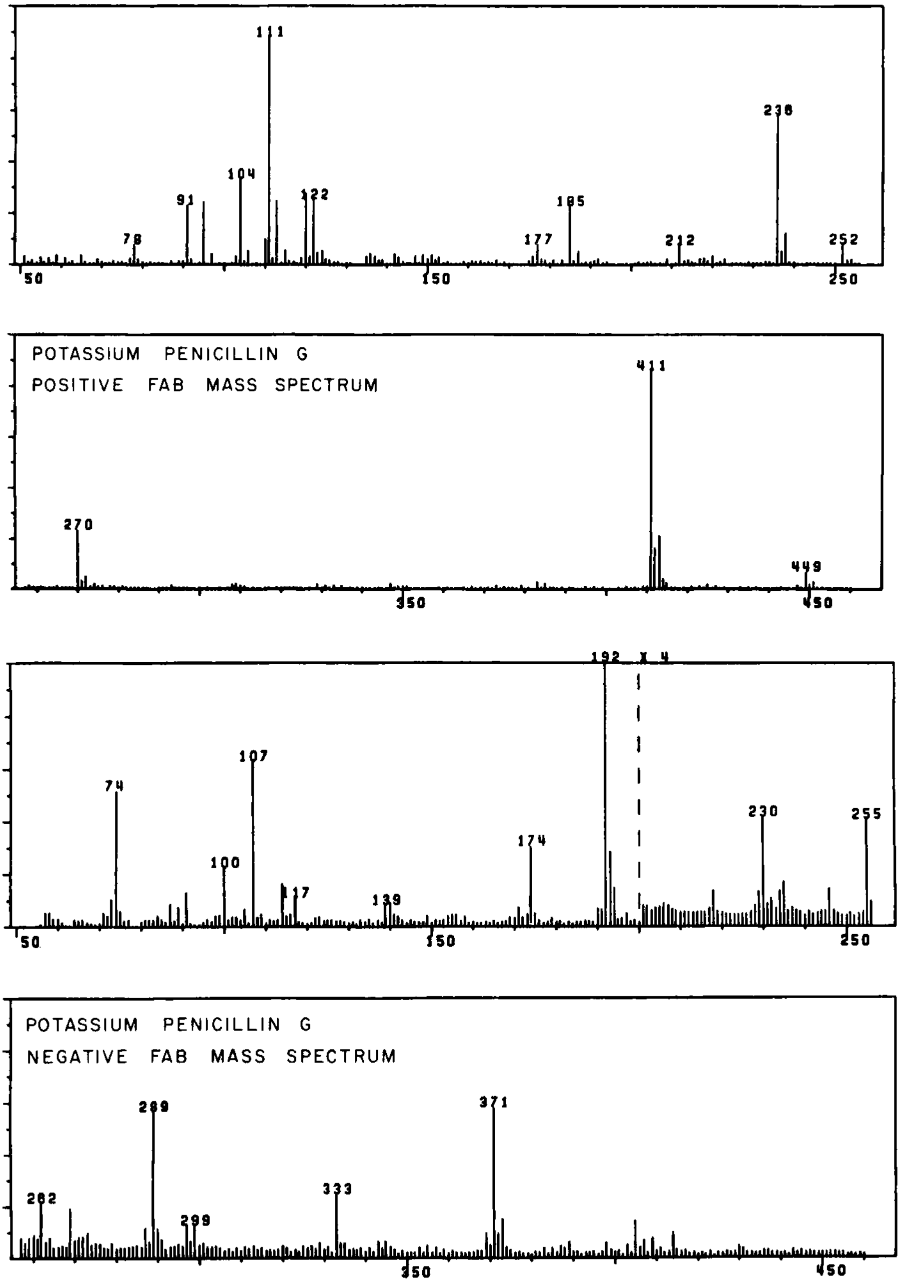


Figure 7. Mass Spectroscopy of Potassium Benzylpenicillin Positive (top) and Negative (bottom) Fast Atom Bombardment Mass Spectra.

Decarboxylation from the $(M+K)^+$ parent accounts for the 367^+ fragment observed in its MS/MS spectrum (top). The dipotassiated fragment at m/z 270 is analogous to its protonated counterpart (195) as shown below. This intense daughter ion is formed by cleavage of the β-lactam ring with oxygen migration.

A similar relationship exists for the 236^+ dipotassiated fragment with its (160^+) protonated analog (196). This daughter ion arises from a reverse 2+2 Diels-Alder cleavage. The remainder is observed in the negative FAB MS/MS spectrum at 174^-.

Decarboxylation from the $(M-H)^-$ parent yields the 327^- fragment. Although the 192^- daughter ion is the base peak in the mass spectrum, its intensity is small in the MS/MS spectrum (bottom). This fragment was assigned (197) as $C_6H_5CH_2CONHCHCHS$, which originates predominantly from the deprotonated molecule of the free acid rather than from the monopotassium salt.

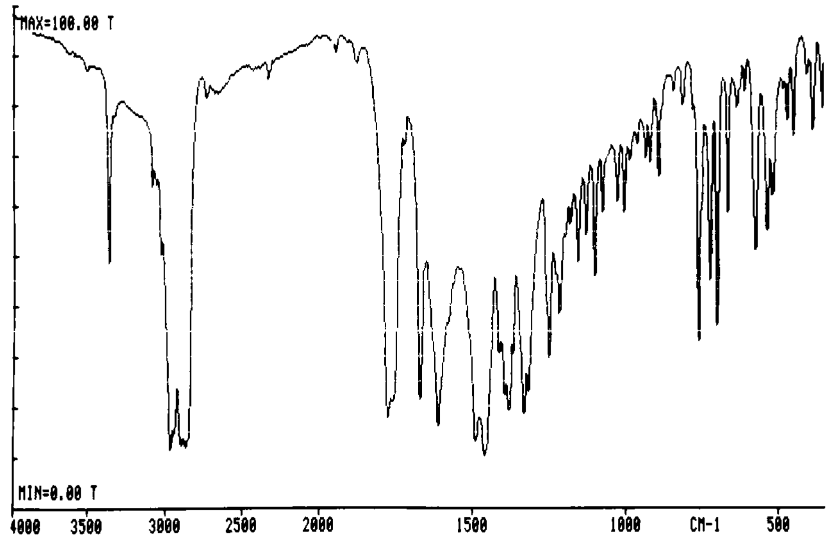
Using electron-impact mass spectrometry, the ion at m/z 334 was found to correspond to the free acid of potassium penicillin G (198). Pyrolysis mass spectrometry was used to characterize some penicillins and cephalosporins (199). Fragments were assigned the following structures: m/e 92, Ø-CH_3; 117, Ø-$CH_2C{\equiv}N$, and 104, Ø-$HC{=}CH_2$. Benzylpenicillin was reacted with BF_3-methanol to yield a derivative amenable to gas-liquid chromatography (see section 5.43). The product was characterized by MS.

2.04 Infrared Spectrometry

Fig. 8 shows the infrared spectra of a commercial preparation of potassium benzylpenicillin using mineral oil and potassium bromide (200). The instrument used was a Perkin-Elmer Model 983 ratio recording (dispersive) infrared spectrometer. Below are the interpretations of these spectra (200,201).

Absorption ($\bar{\upsilon}cm^{-1}$)	Assignment
1772	β-lactam
1666	Amide, I
1486	Amide, II
1606	Carboxylate $-_{as}$ CO_2^-

Mineral Oil Mull



KBr Pellet

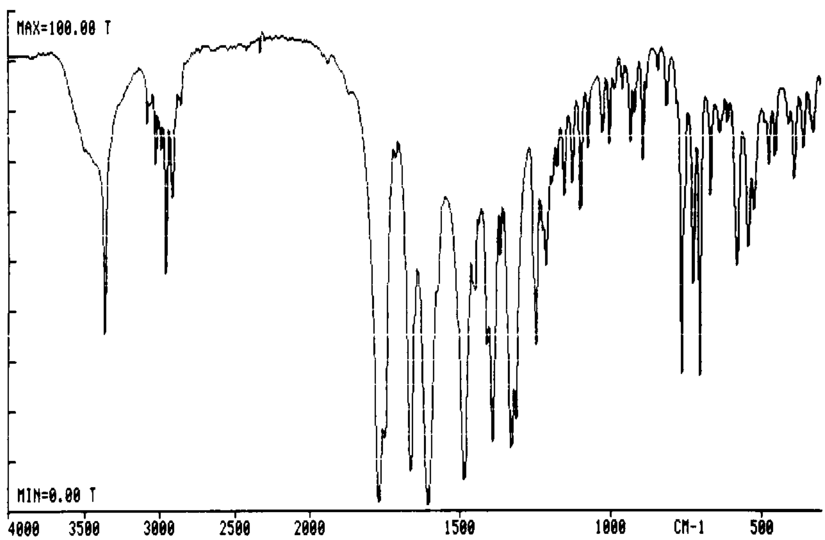


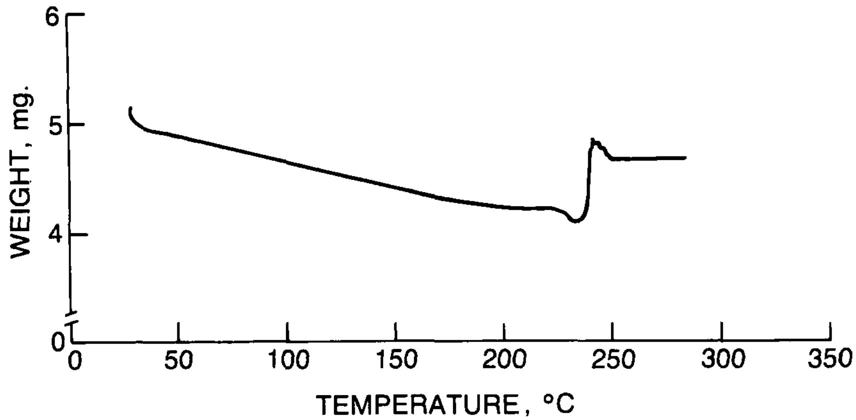
Figure 8. Infrared Spectra of Potassium Benzylpenicillin.

The results are similar to those found in a study of the IR spectra of penicillins (202). The IR spectra of various derivatives and 6-aminopenicillin were obtained and correlated with structure (203).

2.05 Thermal Analysis

A sample of a commercial preparation of potassium penicillin G started melting with decomposition at 218°C (204) using the USP procedure for the melting range of class Ia compounds (205).

Thermogravimetric analysis (206) of potassium benzylpenicillin, using a heating rate of 20°C/min, showed no loss in weight up to 150°C.



Differential thermal analysis of potassium penicillin G gave an exotherm with decomposition at 235-240°C, using a heating rate of 15°min. This is in good agreement with differential scanning calorimetry which also yielded an exotherm with decomposition at 237°-242°C (206).

Thermograms of penicillin and stearic acid, alone and mixed together, were used to demonstrate the formation of an unstable mixture (207). Thermal conductivity and Prandtyl numbers (208,209) for water-butanol solutions of benzylpenicillin were calculated.

2.06 Microscopy and Particle Size

A commercial preparation of potassium penicillin G was found to contain rectangularly-shaped crystals 1 to 3 μm wide and 4 to 25 μm long (206). There was no visual evidence of polymorphism.

Particle size was determined after dispersal in acetonitrile using a Malvern Model 3600E particle size analyzer. The table below gives the percentage of penicillin *vs* size ranges.

Microns	Percent Penicillin
564 - 262	1.6
262 - 160	4.4
160 - 113	6.3
113 - 84	7.5
84 - 65	8.8
65 - 50	9.8
50 - 39	10.4
39 - 30	11.3
30.3- 23.7	11.8
23.7- 18.5	8.8
18.5- 14.5	5.9
14.5- 11.4	5.3
11.4- 9.1	3.4
9.1- 7.2	1.9
7.2- 5.8	1.4

2.07 Surface Area

As measured by nitrogen gas adsorption (206), the surface area of one lot of potassium penicillin G was 0.78 m^2/g.

The surface area was determined to be 0.5 m^2/g for potassium benzylpenicillin (210). After grinding in a jet mill it increased to 2.1 m^2/g, using gas permeability with a PSKh-4 instrument.

2.08 Cohesion

Cohesion (stickiness) was measured by determining the resistance to breakdown of a cylinder of compressed antibiotic powder (210). Potassium benzylpenicillin was 0.52 g/cm^2 before grinding and 11.7 after grinding in a jet mill. The respective measurement errors are 11% and 20%.

2.09 Hydration

The crystals are not solvated with water, based on the thermogravimetric and differential thermal analyses previously described.

2.10 Polymorphism

There is weak evidence for polymorphism from infrared spectrometry and no evidence from x-ray diffraction studies.

3.0 Spectrometry in Solution

3.1 Nuclear Magnetic Resonance Spectrometry (NMR)

3.11 ^{1}H-NMR

Figure 9 is the 270 MHz proton NMR spectrum (211) of potassium benzylpenicillin in D_2O obtained on a JEOL FX-270 NMR spectrometer using a 5 mm C/H dual probe at 30°. The HDO peak at 4.70 ppm from DSS was used as reference. The interpretation of the spectrum is given below.

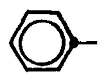
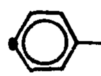
Chemical shift (PPM)	Number of Protons	Assignment
δ 1.47 (s), 1.50 (s)	6	CH_3's
δ 3.47, 3.56(ABq, J=14 Hz)	2	CH_2
δ 4.22 (s)	1	N-C<u>H</u>-$COO^{\ominus}$
δ 5.43 (d, J = 4 Hz)	1	lactam C-H's
δ 5.47 (d, J = 4 Hz)	1	lactam C-H's
δ 7.2 (m)	5	Aromatic

The results are similar to those found in a study of the identification of penicillins and cephalosporins (212). The nuclear Overhauser effect helped elucidate the three-dimensional conformation. Stereochemistry of various derivatives were studied using ^{1}H and ^{13}C NMR spectroscopy (213) and with lanthanide-induced shifts (214). Metal complexation depends on pH (215). A study of the 100 MHz proton magnetic resonance spectra of benzylpenicillin in various solvents that cleave non-covalent bonds (216) showed significant shifts, supporting the hypothesis of self-association previously introduced to explain concentration dependences of ^{1}H NMR spectra (217).

Penicillin G was found to interact with guanosine using 250 MHz NMR and various solvents (218). Binding to bovine serum albumin was studied by 100 MHz NMR, and the literature summarized (219). Spin-echo 400 MHz ^{1}H NMR was used to study the metabolism (220). The kinetics of spontaneous first order degradation of benzylpenicillin in aqueous solution to penicilloic acid gave $k_1 = 0.7 \times 10^{-2}\ h^{-1}$ (221). The conversion of penicilloic acid to labile secondary products followed first kinetics ($k_2 = 6 \times 10^{-2}\ h^{-1}$) using computer simulation.

3.12 ^{13}C-NMR

Figure 10 depicts the ^{13}C-NMR spectrum of benzylpencillin in D_2O also using a JEOL FX-270 Fourier transform NMR spectrometer (211). The reference was *p*-dioxane at 67.6 ppm from TMS for the assignments tabulated below (*cf*. reference 222).

Chemical Shift (PPM)	Assignment
175.6, 175.1, 174.5	C = O's
135.5	
130.4, 129.9	
128.3	
74.2	N-CH-COO$^{\ominus}$
67.7	N-CH-CH-S
65.5	$>C<^{CH_3}_{CH_3}$
59.0	N-CH-CH-S
43.1	CH_2
31.9, 27.6	CH_3's

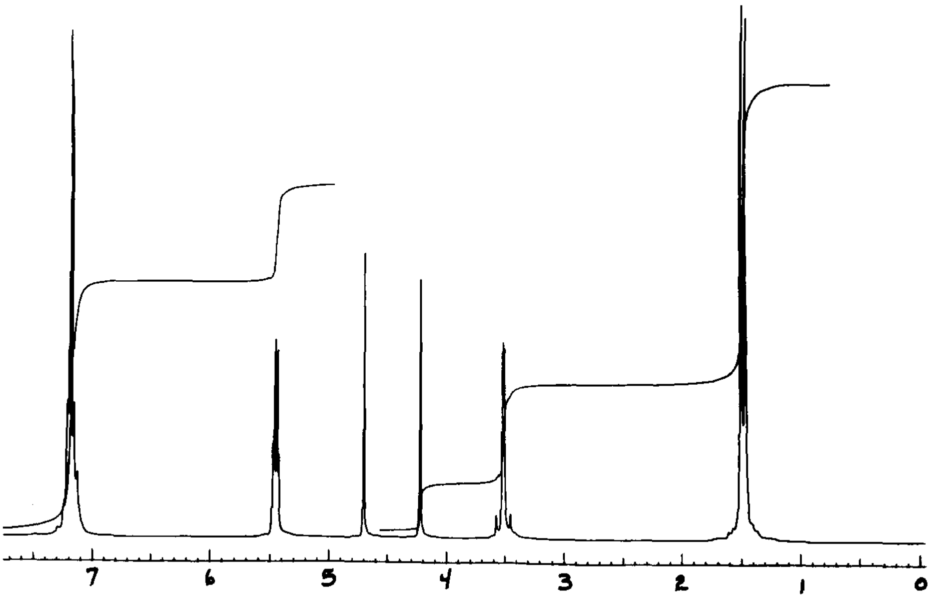


Figure 9. 270 MHz ^{1}H-NMR spectrum of Potassium Benzylpenicillin in D_2O. See text for details.

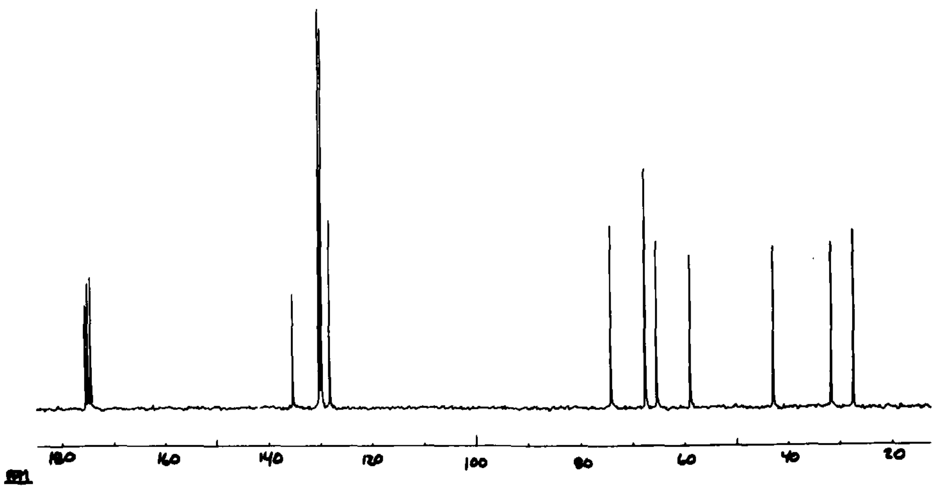


Figure 10. ^{13}C-NMR Spectrum of Potassium Benzylpenicillin. See text for details.

^{13}C-NMR spectrometry was used to study the structures of a series of penicillins (223), penicillins and cephalosporins (224,225) and penicillin derivatives (226). These papers also summarize prior NMR studies.

3.13 ^{15}N-NMR

Although ^{15}N-NMR spectrometry produces sharp resonances, because of its spin of 1/2 its sensitivity is 1.04×10^{-3} that of the proton. In addition, its lower natural abundance (0.37%) results in a sensitivity 3.8×10^{-6} that of the proton. To optimize signal strength, a 2.5 *M* concentration of the soluble methyl ester was used. (Other penicillin and cephalosporin derivatives were also studied.) Two thousand transients were obtained in 1.8 hours to give a S/N ratio of 10. Natural abundance spectra gave ^{15}N chemical shifts relative to an ammonium chloride reference of 134.30 ppm for the lactam and 85.13 ppm for the amide (226). These results are in exact agreement with the ^{15}N-NMR chemical shifts that were obtained in 1,4-dioxane (224). The potassium salt of benzylpenicillin gave shifts of 143.7 and 90.7, respectively, in water, and 142.6 and 86.6 in dimethylsulfoxide using 1.2 to 2 second repetition rates over 6 to 16 hours, also using ammonium chloride.

3.2 Ultraviolet Spectrometry

Figure 11 shows the ultraviolet spectrum of a commercial preparation of potassium benzylpenicillin at a concentration of 2.738×10^{-3} *M* in water, obtained with the aid of a Perkin-Elmer Model 320 Spectrophotometer (227). At 262 nm the ε value is 164 and at 256 nm, $\varepsilon = 251$. Using traditional nomenclature, the E (1%, 1 cm) values are 4.41 and 6.73, respectively. These data are in generally good agreement with previous results (228).

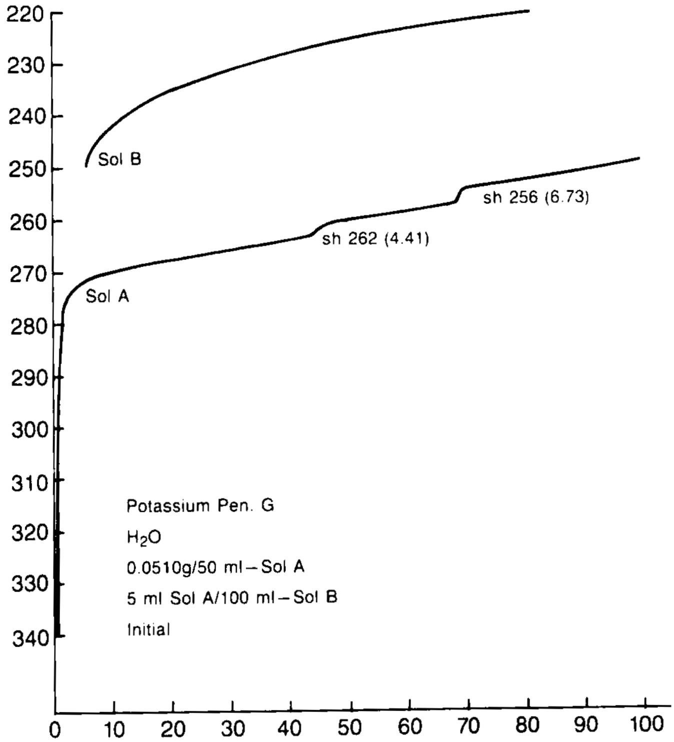


Figure 11. UV Spectrum of Potassium Benzylpenicillin.

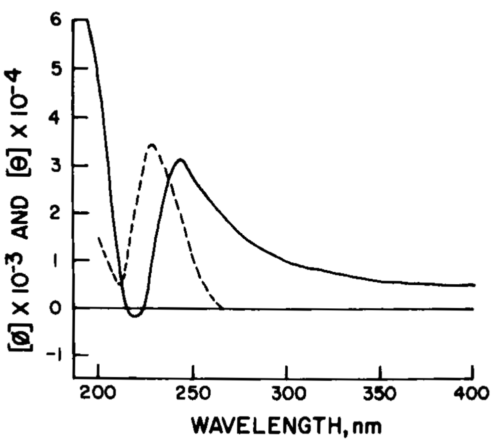


Figure 12. The optical rotatory dispersion (——) and circular dichroism (---) spectra of potassium benzylpencillin in water at pH 6.5. Reprinted here with the permission of the authors and Academic Press.

3.3 Optical Rotatory Dispersion and Circular Dichroism Spectrometry

Figure 12 shows the ORD and CD spectra of potassium benzylpenicillin in water at pH 6.5 (229) as reproduced with the permissions of the author and publisher. Two maxima are visible, one at 230 nm and one below 200 nm. ORD is more informative than CD. The β-lactam functionality produces the 230 nm positive Cotton effect, because 6-aminopenicillanic acid exhibits a similar spectrum, and hydrolysis of the β-lactam bond leads to the loss of this absorption (*cf.* Methods of Analyses, Section 5). The β-lactam (amide) function lacks the ground-state symmetry of the ketone groups. A further complication of the spectra was induced by interaction with the *n*-electrons of the sulfur atom and lack of planarity of the bicyclo ring system. The $[\phi]_{max} \times 10^{-3}$ was 2.92 at 243 nm and $[\theta]_{max} \times 10^{-4}$ nm was +3.14 at 234 nm in 0.1 *M* citrate buffer, pH 6.5 (229,230).

4.0 Bulk Solution Properties

4.1 Solubilities in Aqueous and Nonaqueous Solvents

Solubilities of a commercial preparation of potassium penicillin G were determined (231) at room temperature in various solvents with about one minute of mixing.

Solvent	Solubility (mg/mL)
Acetonitrile	0.1
Chloroform	0.2
Dimethylsulfoxide	>100
Ethanol	10
Hexanes	0.2
Isopropanol	<0.04

Table Continued

Solvent	Solubility (mg/mL)
Methanol	>100
Methylene chloride	<0.04
Methanol-water (1:1)	>100
0.9 *M* NaCl	>100
0.1 *M* HCl	>100
Aqueous buffer, pH 2	>100
Aqueous buffer, pH 4	>100
Aqueous buffer, pH 7	>100
Aqueous buffer, pH 10	>100
0.1 *M* NaOH	>100

4.2 Partition Coefficients

Apparent partition coefficients P_{app} were determined (232) in octanol-water at various pH values at 37° with the help of spectrophotometry at 260 nm, and are summarized below. The intrinsic partition coefficient for the unionized form, P_u was

pH	P_{app}
4.86	0.37
5.07	0.32
5.15	0.23
5.47	0.15

1.70 in octanol. In 2-methylpropanol, the intrinsic partition coefficient for the ionized form was -0.30. The salt form effects apparent partition coefficients.

Using thin-layer chromatography, partition coefficients were calculated for benzylpenicillin

distributed in various amounts of acetone in an aqueous, sodium acetate - veronal buffer, pH 7.4 using siliconized silica plates (233). Benzylpenicillin was visualized using an alkaline solution of $KMnO_4$. Hansch $\Sigma\pi$ values were also obtained. However, the silica gel plates may have exerted a buffering effect on the system.

Instead, *n*-octanol-impregnated microcrystalline cellulose tlc plates were used with a developing solvent of 0.5 *M* 6-aminohexanoic acid (pH 3.5) saturated with *n*-cotanol (234). Benzylpenicillin was visualized on wet plates using NH_3 vapor, and spraying with 10% acetic acid in acetone followed by starch-iodine solution. Log P, the partition coefficient of the organic acid was calculated to be 1.76. This value agrees well with the calculated log $P_{octanol}$ results of 1.72 obtained using high-performance liquid chromatography with an octadecylsilane column, a mobile phase of 0.035 M ammonium chloride-aqueous methanol solution adjusted to an apparent pH of 7.4, and detection at 254 nm (235) for benzylpenicillin partitioned between *n*-octanol and water.

Serum protein binding was found to correlate to partition coefficients of the penicillins (236). Partition coefficients were determined in an organic phase of chloroform and an aqueous phase containing tetrabutylammonium ion and *n*-dodecylamine to form ion pairs (237).

4.3 Ionization

Almost all pencillins and cephalosporins are ionized at physiological pH, making knowledge of the predominant species important in the design of suitable β-lactam antibiotics and their dosage forms. In addition, binding of ^{14}C-benzylpenicillin to such micro organisms as *Staphylococcus aureaus* is a function of pH (238). The pK of benzylpenicillin at 25° and in water at a concentration of 0.0099 *M* was 2.73 ± 0.03 and for 0.0093 *M* was 2.71 ± 0.05 using titration with 0. *M* HCl (239). Titration with 0.01 *N* iodine of benzylpenicillin in aqueous solution at 60°

gave a pK of 2.78 (240). Potentiometric titration was used to determine that the apparent pK value for penicillin G, at 20° and ionic strength 0.15, in 20% methanol was 3.82 and in 30% methanol was 4.10 (241).

4.4 Molecular Aggregation (Self-Association) and Critical Micelle Concentration (CMC)

Potassium penicillin G is capable of self-association in aqueous solution via non-covalent bond formation (242). Light-scattering properties of aqueous and 0.15 *M* and 0.5 *M* potassium chloride solutions indicated the existance of dimers and trimers at critical micelle concentrations, respectively, of 0.32 and 0.30 mol/kg. (The α values of 0.7 imply that most of the monomers constituting the micelle are not in close association with their counterions.) These results agree well with ^{1}H nmr studies (217) from 0.01 *M* to 1 *M* at 30° that indicated a CMC of 0.275 and 0.251 *M*, respectively, for the aromatic and methylene protons. The benzylpenicillin ions appear to aggregate in aqueous solution primarily through hydrophobic interactions of the benzyl side chains. A CMC of 0.25 *M* was also found (243) using cryoscopic and dye-solubilization methods. Unpublished studies using an analytical ultracentrifuge also showed aggregation (244).

Potassium penicillin G also interacts with lipids, such as phosphatidylcholine and lysophosphatidylchloride, as shown by viscosity studies (245). This also indicates that hydrophobic interactions can exist.

5.1 Methods of Analysis

5.1 Compositional Analysis

5.11 Elemental Analysis

Elemental analysis (246) of a commercial preparation of potassium penicillin G gave the following contents, in percent, C, 51.43 (51.59); H, 4.62 (4.60); N, 7.52 (7.52) S, 8.44 (8.61) and K, 10.54 (10.50). The value for sulfur is in excellent agreement with the previously determined content of 8.47 (247).

5.12 Water Content

Water content was determined (248) by mixing 5 to 200 mg with 1 to 5 mL of dehydrated isopropanol and centrifuging. The supernatent (3 μl) was injected into a gas chromatograph. Potassium benzylpenicillin yielded a value of 0.11% (detection limit 3 x 10^{-7} g, linear range 0.20 to 0.2%). In another laboratory, automated stop-flow Karl Fischer titrimetry (249) gave a value of 0.42% (relative standard deviation 0.034%, n=5). These differences probably reflect differences between samples. When gas chromatography (250) was used to analyze residual acetic acid, 0.003% was found (recovery of 99%).

5.2 Titration

The analysis of penicillins and their formulations using titration methods has been known and practiced since 1946 (251) when Alicino utilized the unreactivity of intact penicillin to iodine, while the hydrolysis product, the penicillinoate, reacts with iodine.

Titriant	Comments	References
pH	pH-Stat alkalimetric method	252
pH	Alkalimetric and complexometric	253
Iodometric	Measure iodine consumption before and after hydrolysis	251
Iodometric	In broth, uses penicillinase	254
Iodometric	Automated, broth	255,256
Iodometric	Automated, sensitive	257
Iodometric	Dosage forms	258
Iodometric	Na metaperiodate and arsenite treatment	259
Hg (II)	Uses untreated and hydrolysed penicillin	260
Hg (II)	Complexes with penicillamine	261,262
Fe (III)	Tablets	263

Titriant	Comments	References
p-Chloromercuri- -benzoate	After penicillinase cleavage	264
3-Bromo-4,4-dimethyl-2-oxazolidinone	Brominating agent, comparison with N-bromosuccinimide	265
Penicillin	Used to determine Ag and Cu (II) using ion specific electrodes	266

5.3 Colorimetric and Spectrophotometric Methods

5.31 Colorimetric and UV Spectrophotometric Analysis

Potassium benzylpenicillin has been determined using ultraviolet spectrophotometry virtually from the first production of purified antibiotic (267,268). The technique advanced to the point of using orthogonal polynomials (269) to determine benzylpenicillin in the presence of its degradation products.

Penicillin G was also indirectly quantified by UV after reaction with thiols (270). Penicillins in deuterium oxide or dimethylsulfoxide solution were quantified on the basis of the IR absorbance of the β-lactam band at about 1760 cm^{-1}. Accuracy was claimed to be ±2% (201) and stability in solution can be monitored.

Below is a tabulation of colorimetric assays for benzylpenicillin. Note that benzylpenicillin has been shown to interfere with the determination of other substances (271,272).

Method or Reagent	Comments	Reference
Hydroxylamine	Automated, compendial	273
Hydroxylamine	Bulk drug + broths	274
Hydroxylamine	In broths	275
Hydroxylamine	In broths	276
Hydroxylamine	Automated, dissolution	277
Hydroxylamine	Automated, tablets + capsules	278
Hydroxylamine	Automated, liquid formulations	279

Hydroxylamine	Intravenous solutions	280
Chromotropic acid	Estimation, identification	281
Chromotropic acid H_2SO_4-HCHO	Characteristic colors for different penicillins	282,283
Copper Sulfate, UV	In broths	284
Copper Sulfate, UV	Various penicillins	285
Copper Acetate, UV	Tablets	286
Arsenomolybdate	In broths, automated	287
Mercuric Chloride	Penicillin + penicilloic acid	288
Mercuric Chloride	Also analyze degradants	289
Mercuric Chloride	Automated, sensitive	290
Mercuric Chloride	Stabilized assay, sensitive	291
Mercuric Chloride	+ Other penicillins	292
Starch-Iodine	In sensitivity discs	293
Rhodamine 6	And other compounds	294
Azure B	Via solvent extraction	295
Methylene Blue	Quantify at 640 nm	296
p-(N-methylamino) phenyl sulfate + Cr (VI)	+ Penicillamine	297
Ferrous Nitrate	Penicillin as reagent	298-300

5.32 Optical Rotatory Dispersion and Circular Dichroism Methods

Changes in optical activity of benzylpenicillin due to hydrolysis of the β-lactam ring by penicillinase were measured using a Cary 60 recording spectropolarimeter with a Cary Model 6001 circular dichroism accessory (301). Since the change in specific rotation at 255 nm, $[\Delta_{\alpha}]_{255\ nm}$ deg/mL g^{-1} dm^{-1}, is 5053.9 and the precision of the instrument is 0.2%, the assay has the potential of being precise. Actual precision was found to be 0.1%, with pH and temperature control. Accuracy was verified using microbiological and iodometric assays (302). The method is limited by the error introduced by mutarotation of penicilloic acids.

5.33 Fluorescence Methods

Although penicillin G is only weakly fluorescent after treatment with 0.1 *M* sodium hydroxide (303), it can be derivatized with 5-dimethylaminonaphthalene-1-sulphonylhydrazine. This permits the assay of both the intact penicillin and its biotransformation product, penicilloic acid (304).

5.4 Chromatographic Methods of Analysis

5.41 High-Performance Liquid Chromatography (HPLC)

This author prefers chromatographic methods since similar compounds are usually resolved from each other due to selective interactions of the analyte with the mobile and stationary phases via weak, non-covalent, bonds. HPLC is preferred by many analysts and regulatory agencies because of its specificity, ease of use and high sample capacity. Fig. 13 shows a chromatogram of potassium penicillin G and potassium phenylacetate isolated from a fermentation broth (305). An octadecylsilane column (12% loaded or covered) was used with a mobile phase of 0.1 *M* phosphate-methanol-acetonitrile (60:40:5) apparent pH 4.15, flowing at 1 mL/min. into an ultraviolet detector set to 220. Penicillins V, F, and K and 6-aminopenicilloic acid do not interfere. Table 2, below, summarizes HPLC methods. Partition coefficients were determined by HPLC (*cf.* section 4.2).

5.42 Thin Layer Chromatography

Table 3, below, summarizes thin-layer chromatographic methods for potassium benzylpenicillin arranged in chronological order. Use of π-acceptors as spray reagents for the detection of penicillin was described (359).

5.43 Gas-Liquid Chromatography

Table 4, below, summarizes the GLC systems developed for bulk benzylpencillin.

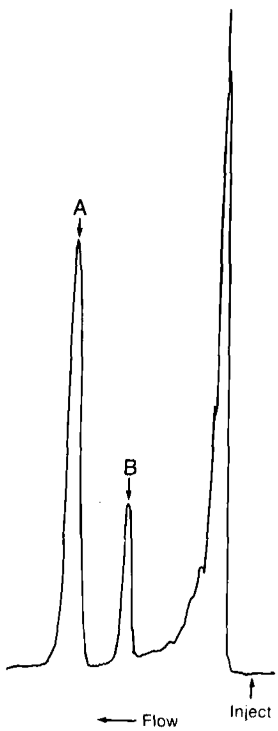


Figure 13. Chromatogram of broth sample with detector set at 220 nm. A, potassium phenylacetate; B, potassium penicillin G. See text for further details.

Table 2. High-Performance Liquid Chromatography of Potassium Benzylpenicillin

Column	Mobile Phase	Detection	Comments	Reference
Bulk or Pure Material				
C18	MeOH-0.5% ammonium carbonate (3:7)	254 nm	Resolves Pen. V.	306
C18	MeOH-0.05 M KH_2PO_4 (35:65) + 0.1% tetrabutylammonium bromide, apparent pH 3.35	254 nm	Resolves other penicillins + degradation products	307
C18	H_2O-MeOH (3:7) containing 0.02 M dicyclohexyl-18-crown 6	220 nm	Describes effects of varying mobile phase. Resolves other β-lactams	308
C18	CH_3OH-H_2O + PIC Reagent B-7	254 nm	Resolves Pen. V + dibenzylethylenediamine	309
C18	Acetonitrile-MeOH-0.01 *M* KH_2PO_4 (19:11:70)	256 nm	Resolves other penicillins	310
C18	MeOH-H_2O (2:1) containing lmM each Na_2HPO_4 and NaH_2PO_4	Postcolumn Rxn	Sensitive, also resolves penicillioates	311
C8	MeOH-0.01 *M* NaH_2PO_4 (7:13)	225 nm	Resolves other penicillins	312

Table 2. HPLC (Continued)

Column	Mobile Phase	Detection	Comments	Reference
C8	MeOH-0.05 *M* phosphate buffer (53:47) apparent pH 3.5-3.3	274 nm or 254 nm	Resolves Pen V. & preservatives	313,314
Anion	0.02 *M* $NaNO_3$ in 0.01 *M* borate, pH 9.15	254 nm	Resolves ampicillin & degradation products	315
Stability				
C18	1% KH_2PO_4-acetonitrile (4:1) pH 4.15	254 nm	Resolves degradation products	316
C18	Acetonitrile-phosphate buffer + 0.008 M tetrabutylammonium chloride, pH 7.5 (3:7)	254 nm	Resolves deg. pro. Mobile phase optimized	317
C18	Acetonitrile-0.01 *M* KH_2PO_4 (1:4), pH 4.1	254 nm	Resolves deg. pro. & other penicillins	318
Anion	0.03 *M* citric acid-0.0067 *M* sodium phosphate buffer, pH 2.7, 37°	254 nm	Resolves deg. pro.	319
Anion	15.4 mL 0.1 *M* citric acid + 7 mL 0.2 M Na_2HPO_4 diluted to 650 mL, pH 3.8	RI	Study stability in acid	320

Table 2. HPLC (Continued)

Column	Mobile Phase	Detection	Comments	Reference
Anion	0.68 g KH_2PO_4 + 0.1 g $NaNO_3$/liter, pH 5.1	254 nm	Resolves deg. pro.	321
Formulations				
C18	0.01 *M* ammonium acetate-acetonitrile (4:1)	254 nm	Penicillin in infusates	322
C18	0.01 *M* KH_2PO_4-acetonitrile (4:1)	240 nm	Effect of excipients (dextrose and sucrose adverse)	323
C18	0.42% KH_2PO_4, 0.6% sodium heptane-1-sulfonate, pH 3.17-acetonitrile (71.5 : 28.5)	258 nm	Controlled-release products	324
C18	MeOH-H_2O-acetic acid (40:60:0.5)	232 nm	Study kinetics decomp. eyedrops	325
Biological Matrices				
C18	0.01 *M* H_3PO_4-acetonitrile (4:1 to 2:3)	220 nm	0.05 ppm in tissues	326

Table 2. HPLC (Continued)

Column	Mobile Phase	Detection	Comments	Reference
C18	0.01 *M* NaH_2PO_4, 0.01 *M* EDTA-acetonitrile (72:28), pH 6.5 (NaOH)	325 nm	Derivatize with imidazole-$HgCl_2$ reagent, broths	327
C18	acetonitrile-pH 7 buffer (23:77)	230 nm	In body fluids	328
C18	phosphate buffer (ionic strength 0.1)-methanol (6:4) pH optimum	Derivatization	In body fluids	329
C18	Gradient: 90% 0.05 *M* Na formate, pH 5-10% formate-acetonile (5:3) to 75:25	240 nm	In urine	220
Misc. and Related Projects				
C8	phosphate buffer, pH 7-MeOH (6:4)	220 nm	Pen. G in broth & 6-aminopenicillanic acid	330
Anion Cation	0.003 *M* $NaHCO_3$/0.0024 *M* Na_2CO_3 0.005 *M* HNO_3	Conductivity	Cation analysis & Pen. G	331
C18	50% MeOH in 0.1 *M* phosphate buffer, pH 4.8; 0.1 *M* H_3PO_4 to 40% acid in 9 min.	254 nm	Sep. procaine, benzathine and Na pen.	332

Table 2. HPLC (Continued)

Column	Mobile Phase	Detection	Comments	Reference
Silica	Acetonitrile- CCl_2H_2 (1:199)	230 nm	Prep. LC of Derivatives	333
C18	15-20% MeOH-0.01 *M* potassium phosphate, pH 7	220, 254 nm	Sep diastereoisomers of penicilloic acids (allergens)	334
C18	Acetonitrile-0.01 *M* sodium phosphate buffer, pH 6.5 (1:4) containing 0.01 *M* EDTA	325 nm	Derivatization with imidazole plus metal salts	335
C18	Acetonitrile-0.05 *M* KH_2PO_4 (17:83) adjusted to pH 4.0 (H_3PO_4)	254 nm	Study epimerization of benzylpenicilloic acid	336

Table 3. Thin-Layer Chromatography of Potassium Penicillin G

Coating	Developing Solvent	Visualization	Comments	Ref.
Silica	Org. Phase isoamyl acetate-CH_3OH-HCO_2H-H_2O (65:20:5:10) or Acetone-acetic acid (95:5)	10% Aq. $FeCl_3$ (20 mL) + 5% aq. potassium ferricyanide (10 mL) + 20% H_2SO_4 (70 mL)	For 10 penicillins	337
Cellulose MW300	0.1 *M* NaCl or 0.3 M citric acid saturated with *n*-butanol			
Silica	$CHCl_3$:isopropanol-H_2O (60:40:4)	iodine-azide	Sep. most penicillins	338
Sephadex G-15	0.25 *M* phosphate buffer, pH 6 containing 0.5 *M* NaCl	Bioautography	Sep. other antibiotics	339
Silica	Acetone-acetic acid (19:1) or isoamyl acetate-CH_3OH-HCO_2H-H_2O (13:4:2:1) (Org.)	UV, then Ref 337 reagent or Bratton-Marshall reaction	Resolves degr. products	340
Polyamide	H_2O-HOAc-isopropanol (50:15:8.5) or H_2O-HOAc-isoprOH-MeOH (50:16:7.1:5)	0.5% Br solution or 0.25% Na fluorescein	Detects other penicillins	341

Table 3. TLC (Continued)

Coating	Developing Solvent	Visualization	Comments	Ref.
Silica	*n*-BuOH-H_2O-EtOH-AcOH (5:2:1.5:1.5) *n*-BuOH-H_2O-AcOH (4:1:1) Acetone-AcOH (95:5) 85% aq. acetone	First 2 *M* NaOH, then iodine-azide, followed by 1% starch	Sep. and detection of penicillins and cephalosporins and their degradation products.	342
Cellulose	BuOH-MeOH-HOAc-H_2O (45:30:9:36)	Bioautography	Detects as little as 0.001 μg in feed and food	343
Silica	Acetone-HOAc (95:5)	See reagent ref 337	For degradation products	344
Silica	Acetone-$CHCl_3$-HOAc (10:9:1)	UV densitometry 230 nm	Quantify in syrups and tablets	345
Silica	Acetone-$CHCl_3$-HOAc (10:9:1)	Ferricyanide (ref 337) then I_2	also degr. products	346
Silica	*n*-BuOH-HOAc-H_2O (12:3:5)	1 mL 2% $PtCl_4$, 0.1 mL 20% KI, 0.1 mL 4% HCl and 20 mL acetone	0.05 μg limit	347

Table 3. TLC (Continued)

Coating	Developing Solvent	Visualization	Comments	Ref.
Silica	$CHCl_3$-MeOH-H_2O (80:20:2.5) or Citrate buffer	UV, 254 nm	Resolves many antibiotics	348
Silica	Plates impregnated with 2% NaAc, adjusted to pH 7.4 barbital. Barbital acetate buffer-acetone (94:6)	Bioautography	as contaminant	349, 350
Silica	BuOAc-AcOH-MeOH-*n*-BuOH-phosphate buffer, pH 7.3 (80:4:5:15:20)	10% Fe Cl_3-2% hexacyanoferrate-HCl (1:2:6)	For various penicillins	351
Silica	BuOH-MeOH-AcOH-H_2O(37.5:25:7.5:16)	Chloroplatinate	In chicken muscle	352
Glass Fiber	BuOH-H_2O-HOAc (4:1:1) or hexane-BuOH-H_2O-EtOH-HOAc (5:10:4:3:3) or hexane-acetone-HOAc (1:9:1) acetone-$CHCl_3$-HOAc (10:9:1)	———	Sep. various penicillins	353
Si	Benzene (caution; replace, if possible, with toluene)-CH_3OH-acetone (15:3:2)	Spray of 50% H_2SO_4 followed by 120°C or 5% $FeCl_3$ in 0.5 *M* HCl or 0.1% aq. fast green (diazotized), drying, then 0.5 M NaOH spray	Seperate and identify various	354

Table 3. TLC (Contined)

Coating	Developing Solvent	Visualization	Comments	Ref.
Silica	$CHCl_3$:MeOH:HOAc (90:8:2) or $CHCl_3$:MeOH:H_2O (20:4:5) or Potassium phosphate, pH 3-washed plates with BuOH-HOAc-H_2O (2:1:1) or H_2O:Na citrate:citric acid (100:20:5) or MeOH or EtOH or H_2O	Bioautography or colorimetry	A classification and identification system for 45 antibiotics is given.	355
Silica	Ethyl acetate	UV, 254 nm	Identify 10 penicillins	356
Silica	Acetone-$CHCl_3$-HOAc (10:9:1)	10% $FeCl_3$-5% K ferricyanide-20% H_2SO_4 (2:1:7)	Contamination	357
Silica	0.5 *M* NaCl or acetonitrile-water (4:1) or EtOAc-CH_3OH-HOAc(20:10:1) or $CHCl_3$-EtOH-HOAc(100:50:7.5) or EtOAc-acetone-H_2O (1:2:1) or isoamylacetate-CH_3OH-HCO_2H-H_2O (13:4:1:2) (upper layer) or acetone-HOAc (95:5) or EtOAc-acetone-HOAc-H_2O (5:2:2:1) or *n*-BuOH-HOAc-H_2O (4:1:1) or *n*-BuOAc-*n*-BuOH-HOAc-0.066 *M* phosphate, pH 6.0 (90:9:25:15) or *n*-BuOAc-*n*-BuOH-HOAc-0.1% EDTA, diNa in 5% NaH_2PO_4 (10:1:6:2)	I_2 vapor	18 penicillins studied in various normal-and reversed-phase mobile phases	358

Table 3. TLC (Continued)

Coating	Developing Solvent	Visualization	Comments	Ref.
Silica Silanized	Buffers are 2 *M* NH_4OAc adjusted to pH 5.0 or 6.2 or buffer pH 6.2-MeOH (17:3) or buffer pH 6.2-MeOH-acetonitrile (7:1:2) or buffer pH 6.2-acetone-EtOH(7:2:1) or buffer pH 6.2-acetone-EtOH(6:3:1) or buffer pH 6.2-MeOH-EtOH(5:4:1) or buffer pH 5-acetonitrile (17:3) or buffer pH 5.0-ethylene glycol monoethyl ether (4:1) or buffer pH5-acetonitrile-ethylene glycol monoethylether (8:1:1) or buffer pH 5-THF(3:1) or buffer pH 5-acetone-ethylene-glycol monoethyl ether (15:3:2) or buffer pH 5-EtOH(3:1) or buffer pH 5-EtOH-ethylene glycol monoethyl ether (15:3:1) or buffer pH 5-acetone (7:3) or buffer pH 5-EtOH-ethylene glycol monoethyl ether (7:1:2) or buffer pH 5-EtOH (13:7) or buffer pH 5-MeOH-acetonitrile (12:3:5) or buffer pH 5-MeOH-EtOH (5:4:1) or 0.3 *M* NaCl in 0.05 *M* potassium phosphate buffer pH 5.6-acetone (2:1) or 0.1 *M* NaCl-acetone (2:1) or 0.05 *M* potassium phosphate buffer pH 6.0-acetone (4:1)	I_2 vapor	18 penicillins studied in various normal- and reversed-phase mobile phases.	358

Table 4. Gas-Liquid Chromatography of Benzylpenicillin

Column	Carrier	T(Column)	Detection	Reference
0.4% SE-52 on acid-washed, silanized Gas-Chrom I (100-120 mesh); 130 cm x 4 mm, Penicillin converted to methyl ester by reaction with diazomethane.	Ar	240°C	Strontium, 800V	360
2% OV-17 on Supelcoport (80-100 mesh); 660mm x 4 mm, conditioned with HMDS to silylate reactive sites. Penicillins silylated within 10 min. with HMDS in pyridine.	He, H_2, Air	275°C	Flame Ionization	361
3% XE-60 on Gas Chrom Q (80-100 mesh) 2 m x 0.32 cm. Pyrolysis unit 20°C/millisec to 875°C.	He	100°C	Flame Ionization	362
5% FFAP on Chromosorb W (AW-DMCS, 80-100 mesh), 3 m x 0.32 cm. Penicillin reacted with BF_3 in methanol.	H_2, Air, N_2	190°C or 140°-230°C at 16° min.	Flame Ionization	363
3% OV-1 on Gas-Chrom Q (60-80 mesh), 2.75 m x 0.63 cm glass. Benzyl-penicillin was derivatized with BF_3 in methanol.	He	50° to 200°C at 16°/min.	MS	363

5.44 Paper Chromatography

Details of several methods for resolving potassium benzylpenicillin are to be found in ref. 338 and 364-366.

5.5 Electrochemical Methods of Analysis

5.51 Electrophoresis

Electrophoresis was used to separate and detect benzylpenicillin mixed with other penicillins in various electrolytes (367,368) in animal tissues (369) and feedo (370) and in food (371).

5.52 Polarography and Related Techniques

Penicillin G was analzyed using potentiometric (372,373 and references therein), polarographic (374-379 and references therein), voltammetric (380) and coulometric (381) methods of analysis. For other electrochemical methods see the following sections on enzyme electrodes and electrophoresis.

5.53 Enzyme Electrodes and Flow Injection

Penicillin-sensitive electrodes were developed with high selectivity based, in general, on the immobilization of a penicillinase on an electrode. Potassium penicillin G could be quantified in fermentation broths and capsule formulations from 3.5 to 1100 μg/mL, with a precision of ±3%, measuring changes in pH as the penicillin was converted to penicilloic acid (382). Changes in pH were measured and shown to be linear with increasing concentrations of penicillins (383,384) in buffered solutions using penicillinase-coated pH electrodes. Care must be taken to allow equilibration to occur to achieve reproducibility and linear sensitivity to concentrations as low as 10^{-4} *M* (385).

An enzyme electrode sensitive to sodium and potassium ions was used to analyze for potassium benzylpenicillin (386), with generally non-linear results.

Using a co-cross-linked penicillinase-albumin layer over a pH-sensitive, field effect transister gave an enzyme electrode generally insensitive to

variations in temperature and ambient pH variation. The sensitivity is approximately 2.5×10^{-4} international units (387).

As many as 150 analyses per hour could be performed using flow injection (388) equipment in conjunction with penicillinase immobilized on a glass surface with glutaraldehyde, and pH monitoring. Penicillin G was determined in pharmaceutical samples in the range of 0.05-0.50 m*M* after dilution.

5.6 Biologically-Based Assays

5.61 Microbiological Assays

The original assays for benzylpenicillin were predominately microbiological (389,390). More recently, microbiological tests were developed to assay penicillin G in broths (391), bulk material (392-395), formulations (396-398), and tissues and body fluids (399-402). There have been many publications describing assays for residual penicillin G in animal tissue and milk (403-410).

The rate of microbial growth was investigated in the presence of various β-lactam antibiotics (411,412), with the resistant organism following apparent first order regrowths. Inhibition of $^{14}CO_2$ release by bacteria was studied (413).

Technical advances resulted in rapid assays based on light scattering (414) and changes in pH (415,416). The factors affecting accuracy were studied (417-423), as was susceptibility (424-428), and the effect of infection (429). Bioassays can be automated (430,431). Unfortunately, space limitations preclude a more complete discussion of this once-essential method.

5.62 Immunoassay

Antibiotic immunoassays (432) were applied to potassium penicillin G to analyze bulk penicillin (433), penicilloyl derivatives (434,435) and penicillins for allergy producing compounds (436,437).

5.63 Enzyme-Aided Assays (Hydrolysis)

Penicillins in mixtures can be quantified on the

basis of different rates of hydrolysis at acid pH with the final measurements performed by UV spectrophotometry (438). β-lactam antibiotics, including potassium benzylpenicillin, could be estimated in biological fluids on the basis of these antibiotics' ability to inactivate the R39 *DD*-carboxypeptidase (439), a rather expensive enzyme.

Penicillin acylase was used to distinguish penicillin G from ampicillin or cloxacillin in bovine urine (440).

The rate of reduction of cytochrome c linked to an enzyme system containing benzylpenicillin and β-lactamase from *Bacillus cereus* was measured at 550 nm to determine activity (441).

Differential UV spectrometry of various penicillins in the presence of β-lactamases, a convenient and widely used method, showed that the changes in absorption were linear with time (442). Kinetic properties of the β-lactamases, like the Michaelis constant and rate constant (V_{max}), could also be measured. The rate of β-lactamase-induced penicillin hydrolysis was also followed by the starch-iodine reaction (443) as well as by iodometric titration (444,445), and acidimetric titration (446). β-lactam-containing antibiotics could be determined within 20 min. using the "Penzym assay" (447).

Hydroxylamine assays were once used routinely for β-lactamase studies; *cf* section 5.31 and reference (448).

5.7 Isotopic Assay

^{14}C-Labeled potassium penicillin G was added to bone cement, pellets molded and the rate of release into aqueous liquids measured using a scintillation counter (449). Subsequent, *in vivo* experiments involved measuring concentrations of antibiotic in the bone adjacent to the antibiotic-impregnated cement.

β-Lactam antibiotics were screened in milk based on a competition between ^{14}C-penicillin and beta-lactam antibiotics for sites on a microbial cell wall that specifically binds β-lactam (450).

5.8 Contamination Methods

Assays were developed to examine benzylpenicillin for contamination, as summarized below.

Contaminant	Comment	Reference
Ethylene oxide	In powders & liquids	451
Cephalosporins	1-2 μg/g	452
Pyrogens	Rabbit test	453
Pyrogens	Limulus (LAL)	454
Particulates	Microscopy	455
Particulates	Scanning EM	456

5.9 Comparison of Methods

Presently, most reliance is placed on HPLC. One company virtually ceased all microbiological testing of β-lactam antibiotics, because of their relatively uncomplicated structures, in favor of chemical testing. However, in the opinion of the author, an HPLC specialist, at least one biological use test is mandatory prior to human use, and safety tests ensure lack of adverse reactions. Various assays involving titration, spectrophotometry with Hg (II) solution and other non-selective methods (457) can only give maximum contents. In the secondary literature may be found assays based on iodometry, hydroxylamine colorimetry, sterility, identity, safety, pyrogenicity, moisture, pH, content, crystallinity, heavy metals content and residue on ignition (458,459).

6. Possible Future Analytical Problems

The rapid excretion and facile inactivation *in vivo* of benzylpenicillin decreases its usefulness as a therapeutic agent. However, these difficulties can be overcome.

The rapid renal excretion ($t_{1/2}$ of 30 min.) can be obviated by large, frequent or continuous intravenous or intramuscular infusion doses. A less wasteful alternative involves formulating potassium penicillin G in waxes or

oil-like liposomes (460) from which, after injection, the penicillin is slowly released. [Slightly soluble salts of penicillin G, like benzathine and procaine are varients of this approach (461)]. Renal excretion can be blocked ~90% by administering probenecid [(p-(dipropylsulphamoyl) benzoic acid] which inhibits active tubular secretion. Concentrations of both drugs need to be monitored.

Inactivation *in vivo* of benzylpenicillin is due principally to the hydrolytic action of β-lactamases (462). The β-lactam clavulanic acid, found by the systematic screening of fermentation products of *Streptomyces clavuligerus* actinomyces, possesses little antibacterial activity but is a potent inhibitor of some β-lactamases of plasmid and chromosomal origin. Administration in combination with clavulanic acid lowers the minimum inhibitory concentrations of many penicillins and cephalosporins. This is an alternative to using synthetic and semi-synthetic β-lactam antimicrobial agents, like methicillin, moxalactam, cefotaxime and the monobactams, which are resistant to hydrolysis by β-lactamases. The amoxacillin-clavulanic acid combination product is called Augmentin, and the potassium clavulanate-ticarcillin injectable is Timentin. Some other β-lactamase inhibitors currently being investigated (463,464) are sulbactam, mecillinam, halopenicillanic acids 6-β-sulfonamidopenicillanic acid sulfones 6-(methoxymethylene)penicillanic acid, acetylmethylene penicillanic acid and 6-aminopenicillanic acid (465-467). These suicide or "Kamikaze" substrates lead to a "dead" β-lactamase (468) via an acyl enzyme intermediate, Fig. 14. The sulbactam-ampicillin combination product is sultamacillin (469). Such formulations of two ingredients require monitoring of bulk, formulations, and body tissue and fluid concentrations similar to those needed for penicillin itself, at least with respect to pharmacokinetic studies.

The recent isolation of biosynthetic genes from *Streptomyces* through molecular cloning techniques should make possible improvements in fermentation yields of penicillin, and, via gene transfer between organisms, novel antibiotics which are "hybrid" compounds (470).

7. Acknowledgements

The author gratefully acknowledge the assistance of the many contributors from the Squibb Institute for Medical Research cited as "personal communication". In

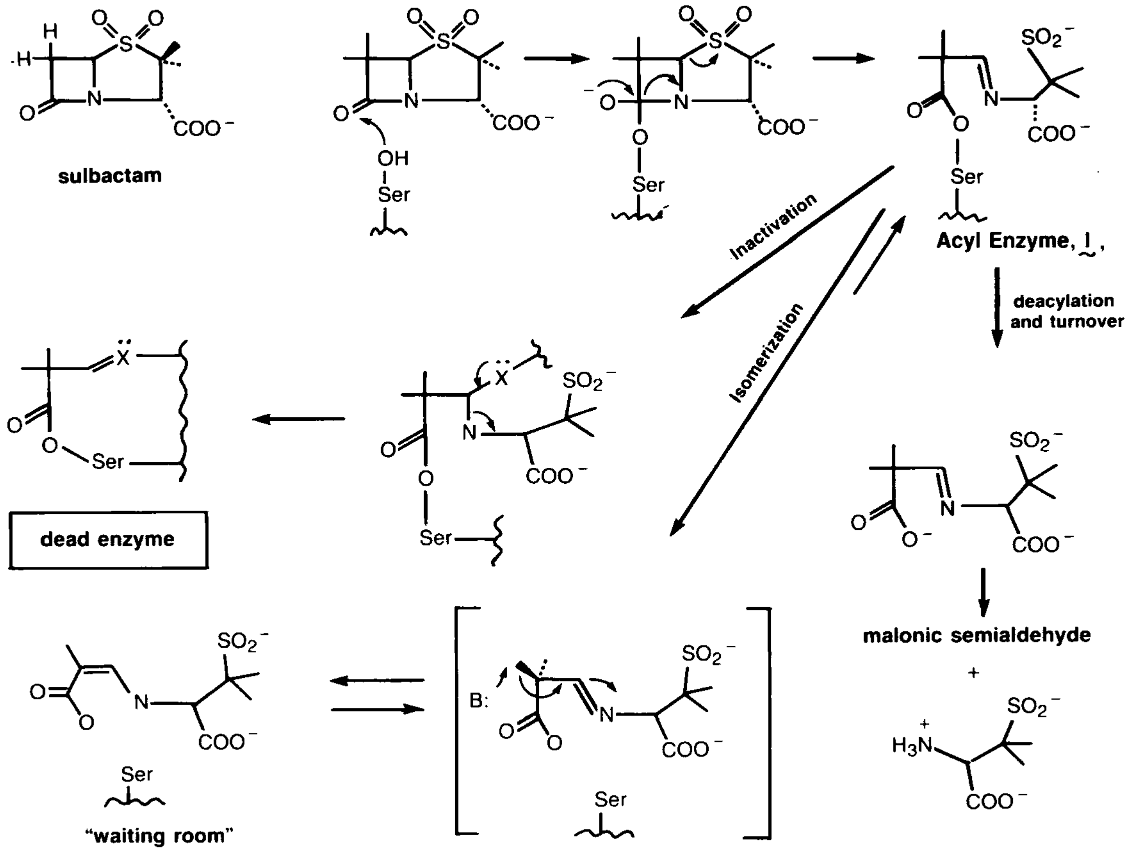


Figure 14. Inhibition of a β-Lactamase by Sulbactam (Redrawn from Ref. 468)

many instances, the information was especially obtained for this summary.

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PIROXICAM

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Nikola Blaževič

1. Foreword, History, Therapeutic Category

Piroxicam belongs to the class of acidic, non-steroid anti-inflammatory (NSAI) drugs. The compound is quite efficient in the treatment of rheumatoid arthritis and other inflammatory disorders in humans /1,2/. The drug is highly potent, has a long half-life of over 3o hours which makes it suitable for once daily dosage /2-4/. It does not have unwanted cardiovascular or central nervous effects /4/.

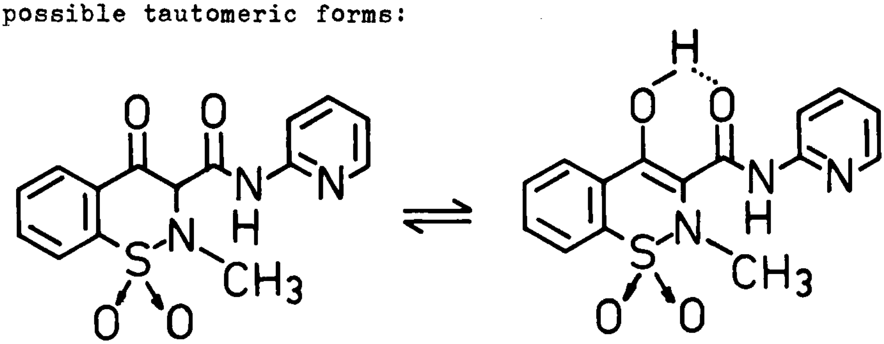
Piroxicam was first developed by Pfizer and Co. about fifteen years ago /5/ and in seventies it entered into medicinal praxis.

Piroxicam is used as an effective analgesic and anti-inflammatory agent in rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and acute pain in musculoskeletal disorders and acute gout /6/. It has been shown to be an effective analgesic in fracture, dental, postoperative and postpartum pain.

2. Description

2.1. Name, Formula, Molecular Weight

Piroxicam is 4-hydroxy-2-methyl-N-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide. There are two possible tautomeric forms:



$C_{15}H_{13}N_3O_4S$ Mol. wt. 331.36

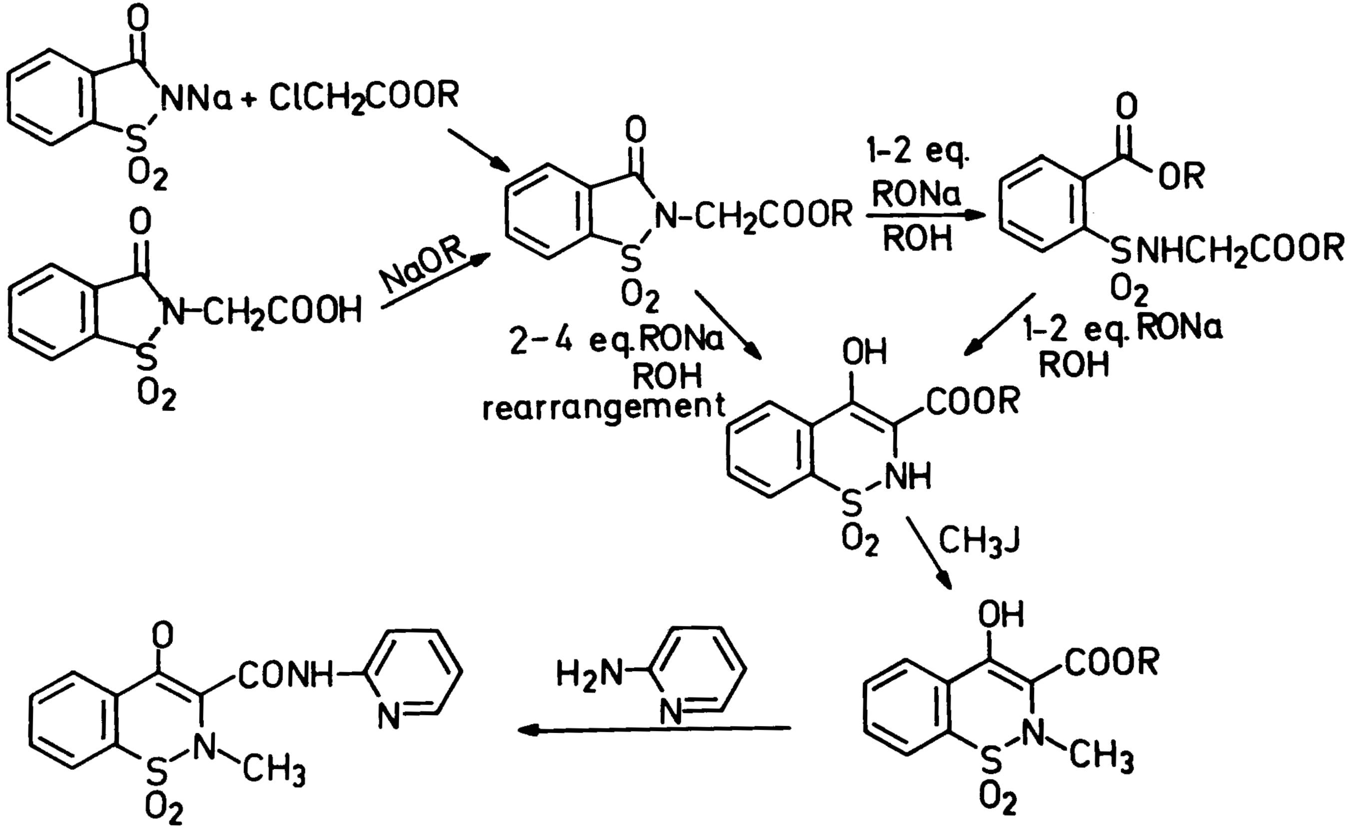
2.2. Appearance, Color, Odor, Taste

Piroxicam is an odorless, colorless crystalline powder of a bitter taste.

3. Synthesis

The most important synthetic pathways to piroxicam are outlined in Scheme 1. The first one /5, 7-9/ starts with sodium saccharin which is alkylated at nitrogen using chloroacetic acid esters /1o/. 3-Acyl-2H-1,2-benzothiazin-4(3H)-one-1,1-dioxide is prepared by the base catalysed

SCHEME 1.



O
NNa + ClCH2COOR
S
O2
O
N-CH2COOH
S
O2
NaOR
O
N-CH2COOR
S
O2
1-2 eq.
RONa
ROH
O
OR
SNHCH2COOR
O2
2-4 eq.RONa
ROH
rearrangement
1-2 eq. RONa
ROH
OH
COOR
NH
S
O2
CH3J
OH
COOR
N
CH3
S
O2
H2N
N
O
CONH
N
N
CH3
S
O2

rearrangement of the 5-membered intermediate comprising alkoholysis followed by a Dieckmann ring closure /11-13/. N-Methyl derivative is readily obtained by reaction with methyl iodide /11, 14/. The product is then treated with appropriate amine in refluxing xylene to obtain the corresponding carboxamide.

4. Physical Properties

4.1. Spectra

4.1.1. Infrared

Piroxicam exists in two different interconvertible crystal polymorphs. Their infrared spectra differ only slightly in fingerprint region, but the band of -NH and -OH stretching which lies at 3385 cm^{-1} in needle form and 3330 cm^{-1} in cubic form is significant /15/. The other characteristic bands /Fig.1./ may be attributed to the following group vibrations: 1635 or 1625 cm^{-1} (stretching of the amide carbonyl), 1525 cm^{-1} (stretching of the second amide band), 1440 cm^{-1} (ν as.-CH_3, Ar-C=C- stretching), 1355 cm^{-1} (ν sym.-CH_3), 1155 and 1070 cm^{-1} or 1050-1070 cm^{-1} (-SO_2-N-), 770 and 730 or 740 cm^{-1} (ortho-disubstituted phenyl). The spectra are taken in KBr discs at Pye-Unicam SP3-200 infrared spectrophotometer.

4.1.2. Nuclear Magnetic Resonance

Proton magnetic resonance spectrum was recorded in $CDCl_3$ solution by Bruker WP80PS spectrometer. It is characterized by one sharp singlet at 2.93 ppm (δ scale) for the N-methyl group, two broad singlets, one at 9.00 ppm (amide hydrogen), and another at 13.20 ppm (hydroxy group). Both hydrogens are exchangeable by addition of D_2O. A multiplet at 6.93-8.50 ppm belongs to aromatic protons and corresponds to four protons from the benzene, and four protons from the pyridine ring, see Fig. 2 /15/.

^{13}C-Nuclear magnetic spectrum was recorded in $CDCl_3$ solution by Jeol FX-100 spectrometer. It is rather complex and difficult to assign properly. The signals of the benzothiazine moiety interfere with those from the pyridine ring (Fig. 3). The values for chemical shifts are presented in Table 1. Note that assignations of signals to C-5 and C-6 might be inverted /15/.

4.1.3. Ultraviolet

The ultraviolet spectra of piroxicam in 0.1 M HCl and methanol recorded by SP8-100 UV/VIS Pye Unicam spectrophotometer are shown in Fig. 4 /15/. These spectra are characterized by two maxima in 0.1 M HCl: at 242 nm (ε = =9700) and 339 nm (ε =22500), and three in methanol: at

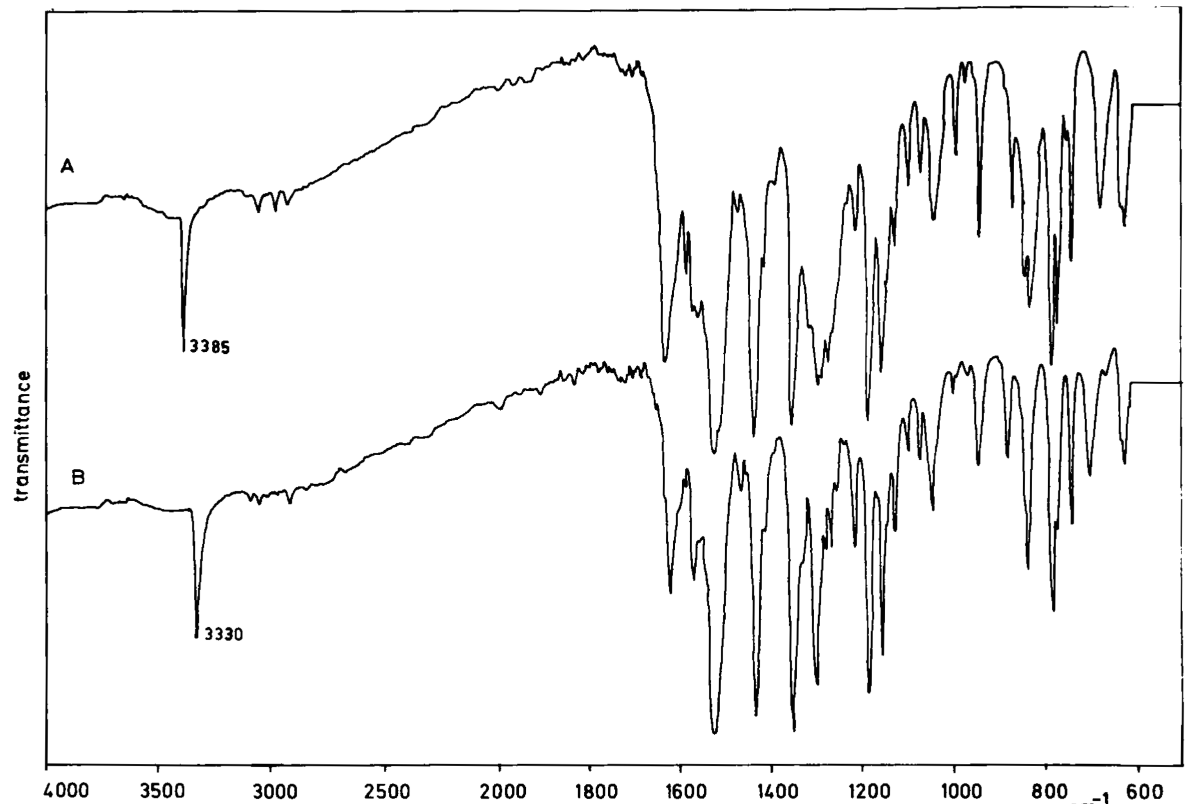


Fig. 1. Infrared spectra of piroxicam in KBr pellets, needle form /A/ and cubic form /B/. Instrument: Pye-Unicam SP3-2oo.

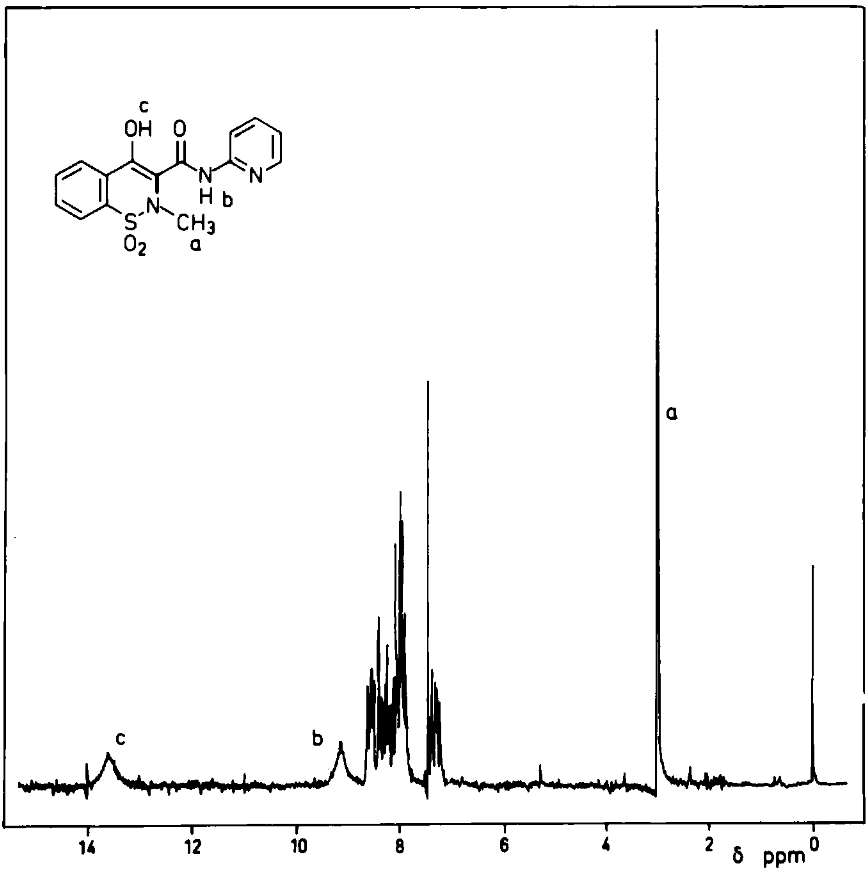


Fig. 2. ^{1}H-Nuclear magnetic resonance spectrum of piroxicam in $CDCl_3$. Instrument: Bruker WP 80PS at 80 MHz.

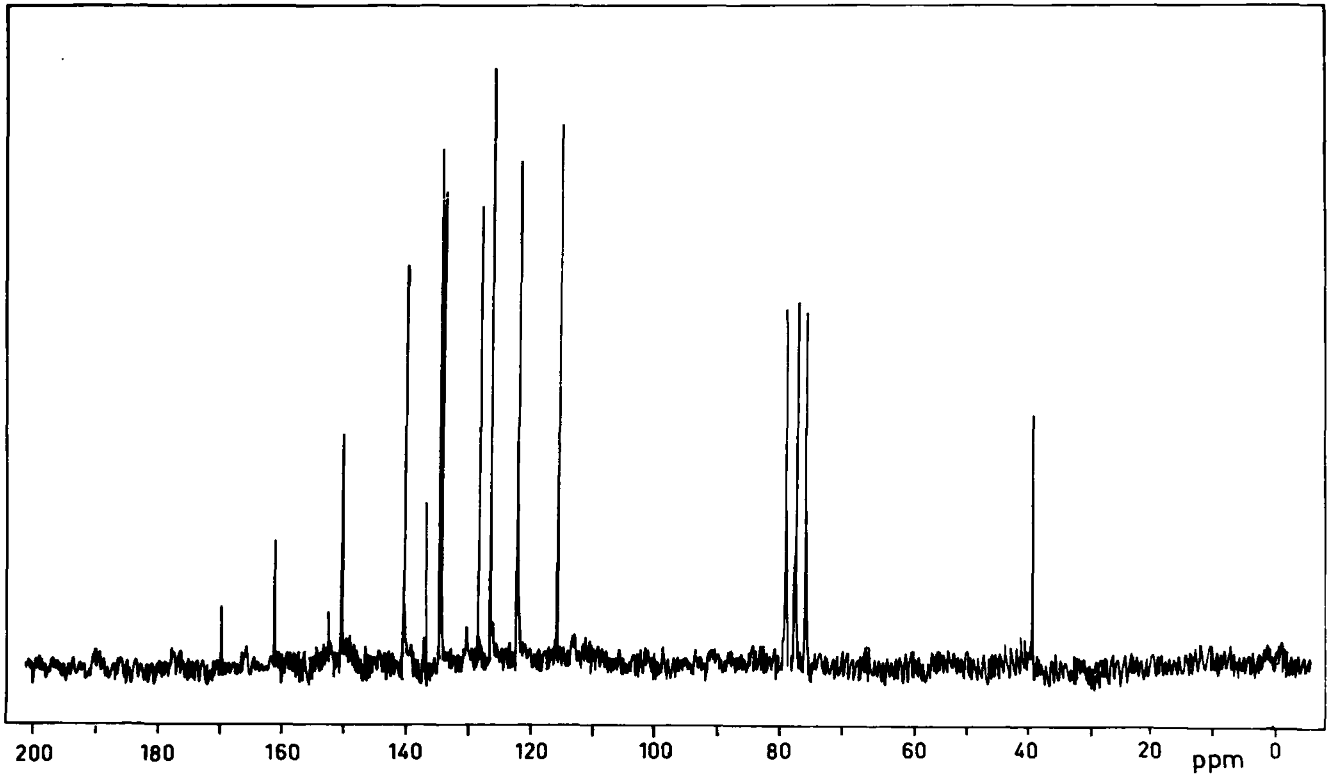


Fig. 3. ^{13}C-Nuclear magnetic resonance spectrum of piroxicam in $CDCl_3$.
Instrument: Jeol FX-loo at 25.o5 MHz.

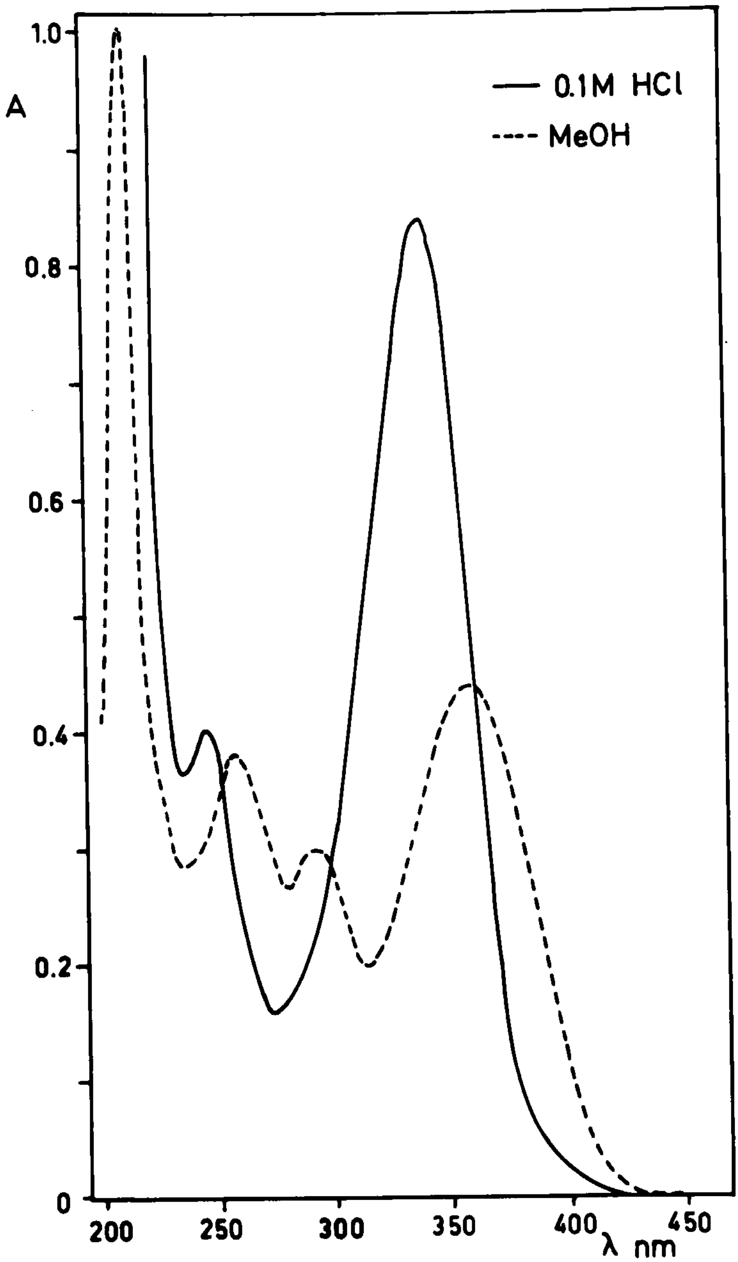
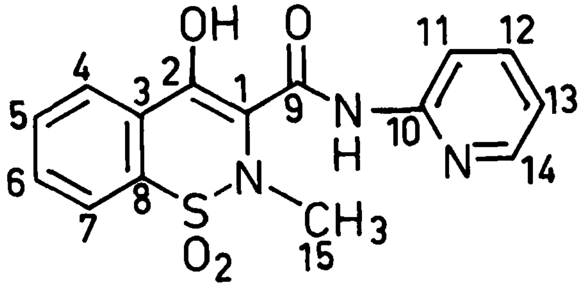


Fig. 4. Ultraviolet spectra of piroxicam.
Instrument: Pye-Unicam SP8-loo.

Table 1. The values for chemical shifts of piroxicam in ^{13}C-NMR spectrum in $CDCl_3$



C-atom	Chemical shift (ppm)	Multiplicity
C- 1	117.7	s
C- 2	159.3	s
C- 3	128.8	s
C- 4	126.6	d
C- 5	132.9	d
C- 6	133.3	d
C- 7	124.2	d
C- 8	135.2	s
C- 9	167.5	s
C-1o	15o.5	s
C-11	115.8	d
C-12	139.o	d
C-13	12o.4	d
C-14	147.2	d
C-15	39.2	q

256 nm (ε=127oo), 29o nm (ε=1oooo) and 358 nm (ε=146oo). The second maximum in o.1 M HCl is suitable for spectrophotometric determination of piroxicam in pharmaceutical dosage forms.

4.1.4. Mass

The fragmentation was studied by Kratos M-25 spectrometer linked to data system DS 5oS, and it is shown in Fig. 5 /15/. A molecular ion M^+=331 is present with 39% intensity relative to the basic ion m/e=173. The pathway of fragmentation is shown in Scheme 2.

4.2. Solid Properties

4.2.1. Polymorphism

Piroxicam exists in two different interconvertible crystal polymorphs with melting points of 196-198°C /needle form/ and 199-2o1°C /cubic form/. The two forms are

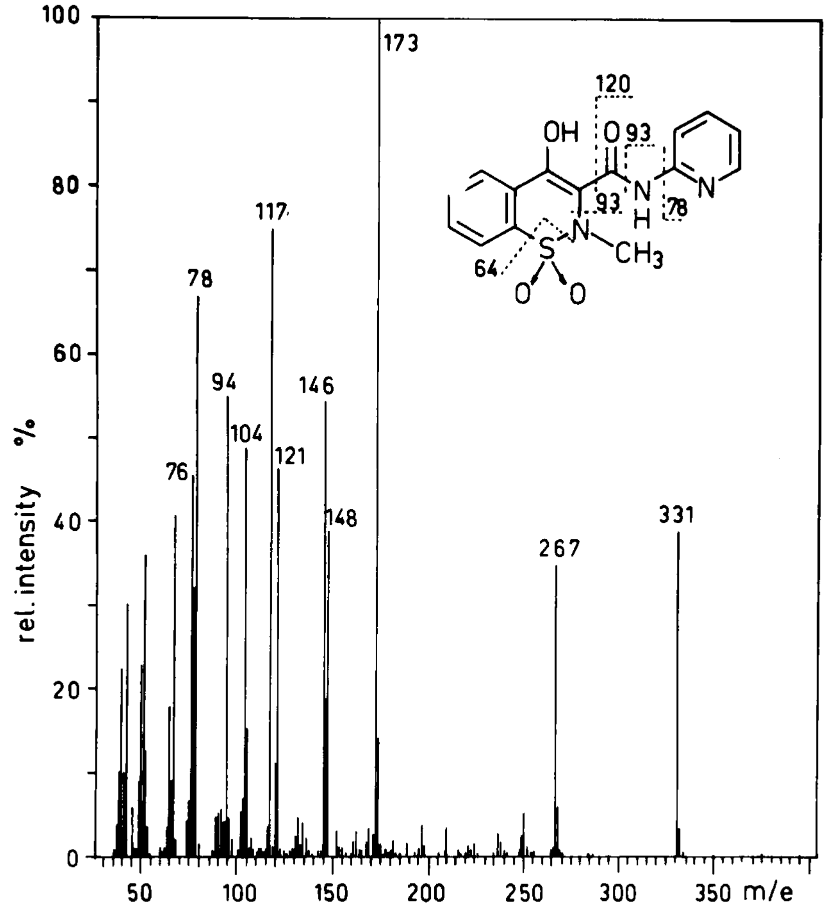
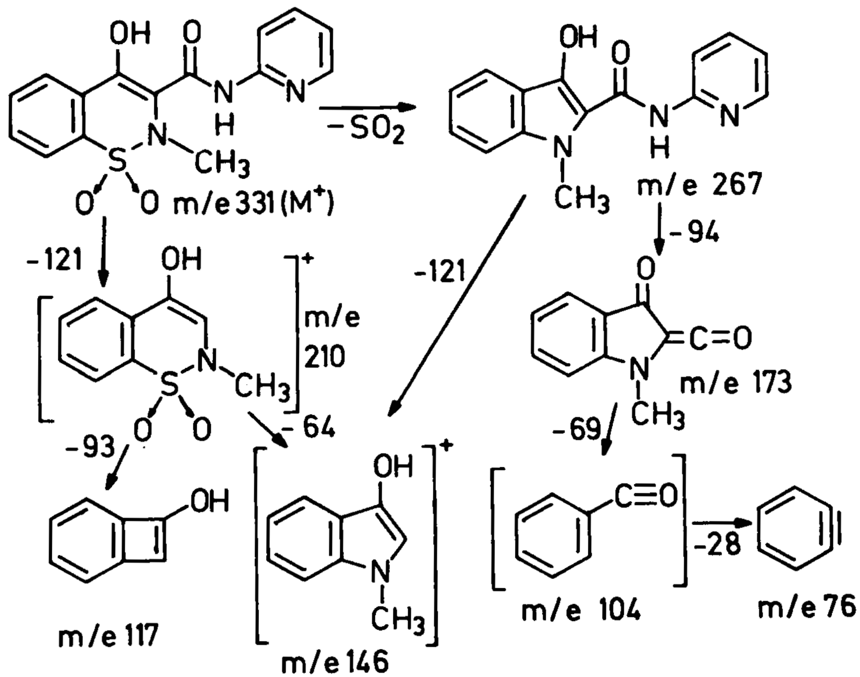


Fig. 5. Mass spectrum of piroxicam at 230°C.
Instrument: Kratos M-25.

SCHEME 2.



OH
O
N
H
N
S
N
CH3
O
O
m/e 331 (M+)
−SO2
OH
O
N
H
N
N
CH3
m/e 267
-121
OH
m/e
210
N
S
CH3
O
O
-121
-94
O
C=O
N
m/e 173
CH3
-93
- 64
OH
OH
N
CH3
-69
C≡O
-28
m/e 117
m/e 146
m/e 104
m/e 76

distinguished by infrared absorption and, even more, by X-ray powder diffraction (refer to the corresponding chapters). There are no data about a possible different activity of the particular form.

4.2.2. Crystal Properties

When allowed to crystallize from an ethanolic solution by fast cooling, piroxicam precipitates as needles, while by slow cooling from the same solution precipitates in cubic form. By crystallization from an aqueous ethanol or aqueous acetone piroxicam crystallizes with one molecule of water which disappears at approximately 12o °C and the melting point does not change.

The measurements of a sample obtained by grinding the crystals of piroxicam at room temperature were taken by a General Electric XRD-6 spectrogoniometer. The value of 2Θ, interplanar distances $d=n\lambda/2 \sin\Theta$, and relative intensities I/I_o based on highest intensity of 1oo, for cubic and needle form of piroxicam, respectively, are given in Tables 2 and 3. The data are obtained with a rate meter T.C. o.5, 1o ooo cps. The corresponding spectra of the X-ray diffraction pattern are presented on Figs. 6 and 7.

The space group of piroxicam (monoclinic, $P2_1/c$) is determined using Weissenberg photographs taken with CuK_α radiation /16/. The cell dimensions were defined from diffractometer measurements: a=7.127(2), b=15.136(7), c=13.949(6) Å, β=97.35(4)°, Z=4, V=1491.15 Å^3, D=1.481 mg m^{-3}, MoK_α/λ=o.71o7 Å, μ=o.244 mm^{-1}/; final R=o.o5o for 2289 observed reflexions $[I > 2\sigma(I)]$. The piroxicam mole-

Table 2. X-Ray characteristics of cubic form of piroxicam

$2\Theta°$	I/I_o	d (Å)
8.7o	71	1o.1649
11.74	24	7.5387
12.3o	37	7.1967
14.62	87	6.o595
16.25	19	5.4552
16.7o	46	5.2o41
17.7o	1oo	4.9o91
2i.oo	21	4.o773
22.56	2o	3.9416
26.72	72	3.3367
27.4o	99	3.2554
27.92	34	3.1959
34.26	19	2.6176

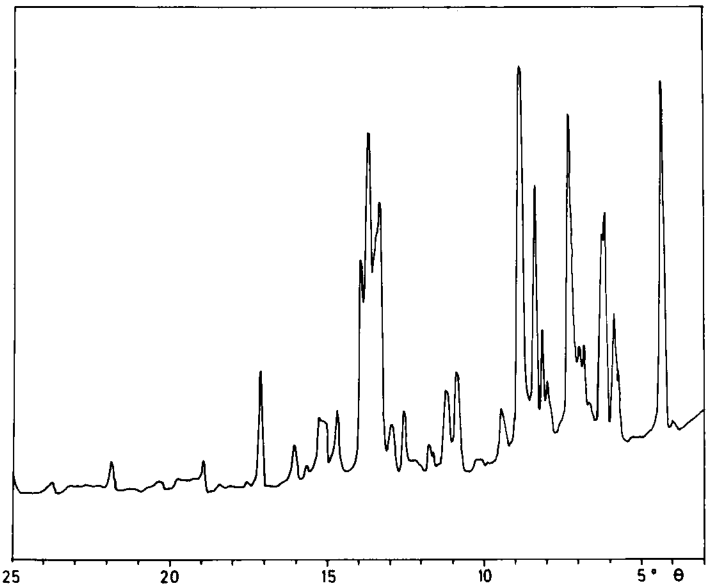


Fig. 6. X-Ray diffraction pattern of piroxicam cubic form. Instrument: General Electric XRD-6 spectrogoniometer.

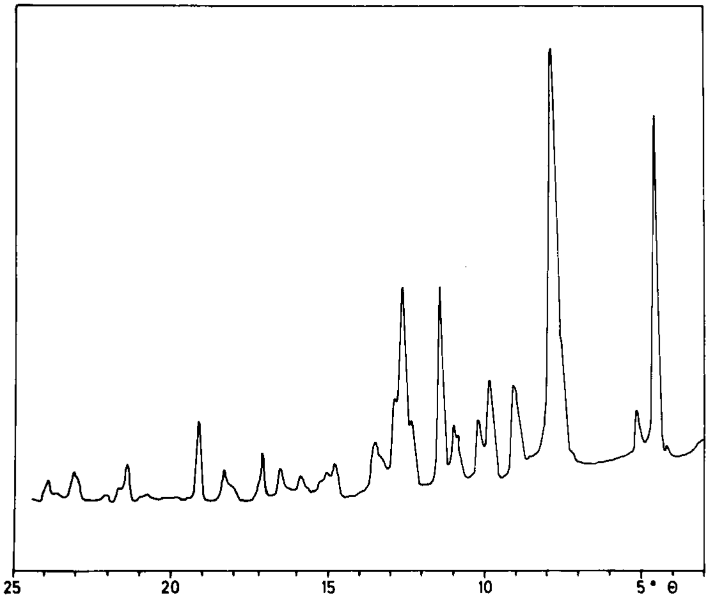


Fig. 7. X-Ray diffraction pattern of piroxicam needle form. Instrument: General Electric XRD-6 spectrogoniometer.

Table 3. X-Ray characteristics of needle form of piroxicam

$2\Theta°$	I/I_o	d (Å)
9.18	23	9.6345
10.60	7	8.3468
15.78	100	5.6166
18.16	17	4.8855
19.64	15	4.5206
20.20	9	4.3965
21.90	10	4.0443
22.85	24	3.8923
25.22	34	3.5316
26.90	0	3.3051
30.24	8	2.3539

cule is not far from being planar (~bc plane). The thiazine ring exhibits a half-chair conformation. An amide group is involved in an intramolecular hydrogen bond with the hydroxy group. It also forms an intermolecular hydrogen bond with the oxygen atom bonded with the sulphur atom, connecting piroxicam molecules in an infinite chain along b axis. The molecular packing is also influenced by van der Waals interaction.

Piroxicam monohydrate, unlike the piroxicam structure, exists in a zwitterionic form /17/, the enolic hydrogen having been transferred to the pyridine nitrogen. Two intramolecular hydrogen bonds are formed by an internal rotation of the neutral structure (between enolate oxygen and hydrogen on amide nitrogen, and between carbonyl oxygen and the hydrogen on pyridine nitrogen). The side chain and the atoms in the thiazine ring are planar. The crystals are prismatic (space group P1) with following cell dimensions: a=12.721(4), b=12.909(4), c=10.481(3) Å, α=99.31(2), β=108.88(2), γ=102.64(2)°, V=1537.9(7) Å^3, Z=4, taken with CuK$_\alpha$ radiation, λ=1.5410 Å, μ=21.3 cm^{-1} /17/.

4.3. Solution Properties

4.3.1. Solubility

Piroxicam is not soluble in water and cyclohexane, sparingly soluble in diisopropyl ether and in toluene, and only slightly more soluble in lower aliphatic alcohols methanol, ethanol and isopropanol . It is soluble in some polar organic solvents such as dimethylformamide (1 g/10 ml), dimethylsulphoxide (1 g/10 ml), chloroform (1 g/20 ml), and somewhat less soluble in dioxane (1 g/

/4o ml), acetone (1 g/5o ml) and ethyl acetate (1 g/8o ml) /15/.

4.3.2. Acidity (pK_a)

Potentiometric titration of piroxicam solution in a mixture of dioxane and water (2:1) gave a pK_a value of 6.3, effected by the enolic hydroxyl group at C-4 /4,7, 18/.

4.3.3. Partition Coefficient

Piroxicam has a partition coefficient of 1.8 between *n-octanol and aqueous* buffer pH=7.4 /4/.

4.3.4. Dipole Moment

The dipole moment of piroxicam was determined in dioxane solution at 2o.o$\pm$o.1 ^{o}C, using a Dipolmeter DM ol (Wiss.-Techn. Werkstätten, D 812 Weilheim). The value found was 3.68$\pm$o.o6 D /15/.

5. Methods of Analysis

5.1. Elemental Analysis

Piroxicam $C_{15}H_{13}N_3O_4S$ (331.36) calc.:

C	54.37 %
H	3.96 %
N	12.68 %
O	19.31 %
S	9.68 %
	1oo.oo %

5.2. Chromatographic Methods

5.2.1. Thin Layer

The identity and purity of piroxicam may be checked by the use of o.2 mm thick Fertigplatte Merck F_{254} and suitable solvents or solvent mixtures, which are with corresponding R_f values of piroxicam listed in Table 4 /15/.

Table 4. TLC characteristics of piroxicam

Solvent	R_f
chloroform	o.1
acetone-cyclohexane	o.56
dichloromethane-ethanol 2o:1	o.58
chloroform-ethanol 1o:1	o.64
acetone	o.68

5.2.2. High Performance Liquid

The chromatogram was made by a Pye-Unicam LC-3-XP chromatograph with UV-LC-3 detector. The column used was

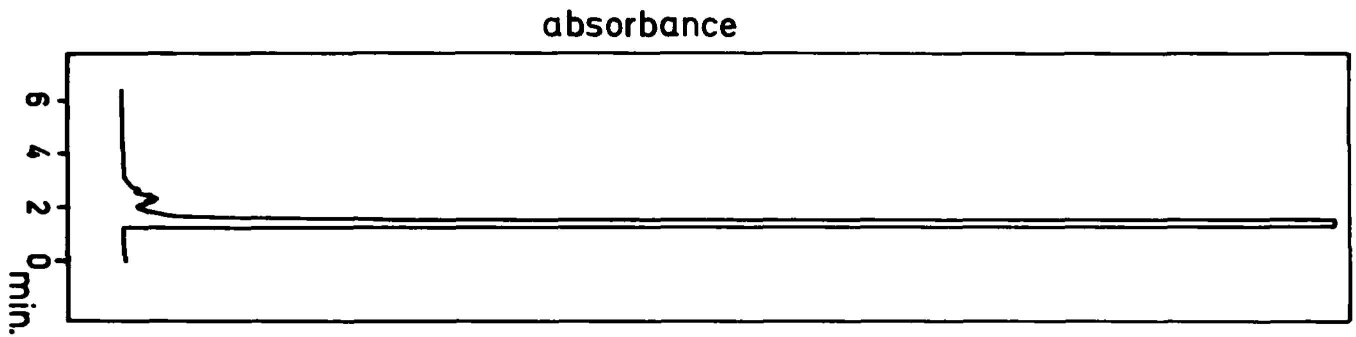


Fig. 8. HPL chromatogram of piroxicam. Instrument: Pye-Unicam LC-3-XP.

Whatman-Partisil PXS 1o/25 PAC. The applied mobile phase was acetonitrile-water-acetic acid 25-75-5 ml, 1.2 ml/ /min . The sample of piroxicam was dissolved in o.1 N NaOH in concentration of 1.o mg/ml and gives one peak on HPL chromatogram /19,2o/, Fig. 8.

6. Stability - Degradation

6.1. Thermal Stability

The sample of piroxicam was kept in a brown powder-glass in the dark at 2o°C and 4o°C. After two years no change in color, smell, taste and shape of crystals could be observed in samples kept at either temperature. TL and HPL chromatograms did not show degradation products. Contents in piroxicam obtained by analytical determinations at various times of exposure are shown in Table 5. /15/.

Table 5. Content of piroxicam kept at two temperatures at various times of exposure

Time months	Content of piroxicam in sample % 2o°C	4o°C
o	99.9	1oo.1
3	1oo.o	99.9
6	1oo.1	99.8
9	98.8	99.6
12	1oo.1	99.7
24	99.8	99.5

6.2. Photostability

Samples of piroxicam o.5 g were filled into colorless 5o ml bottles and were irradiated for 72 hr with light of 3oo-83o nm at 3o±o.5°C. At 24 hr intervals, some of the samples were examined for changes in appearance, smell and taste. Piroxicam contents were determined by HPLC /15/. The results are presented in Table 6.

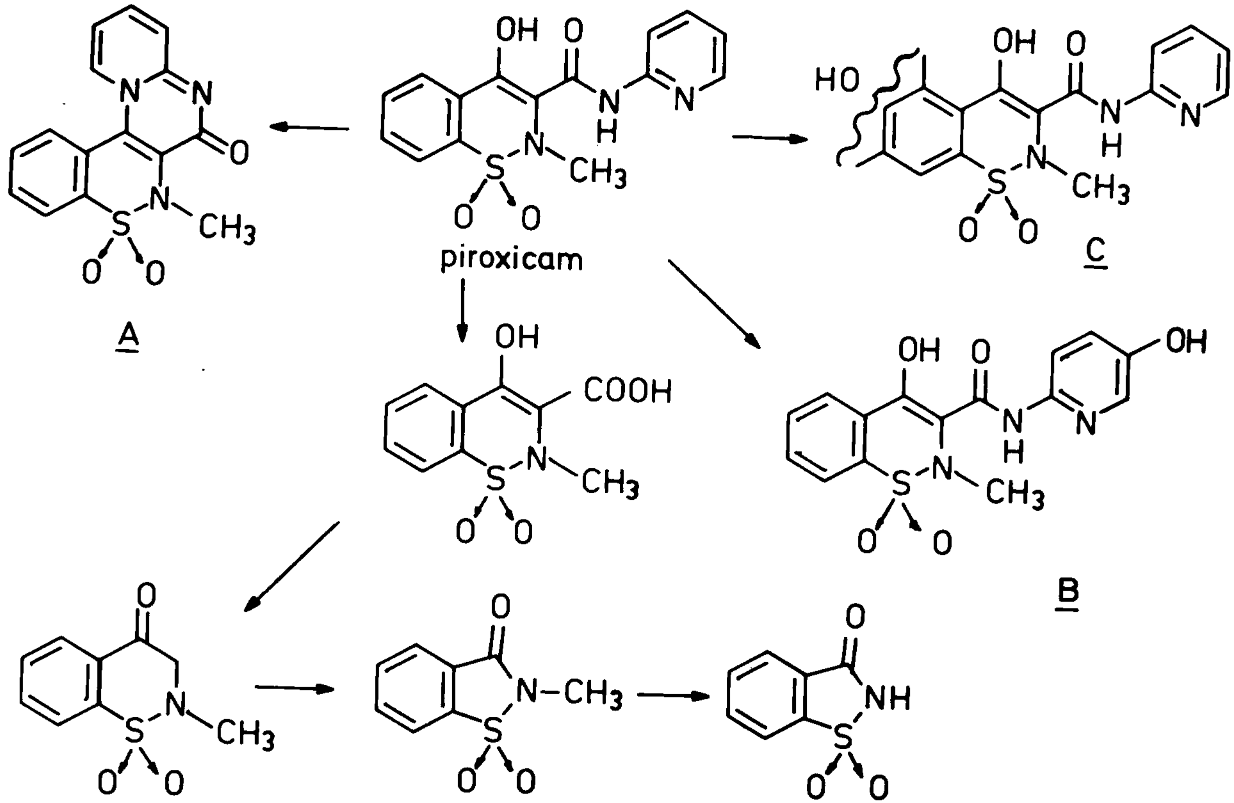
Table 6. Photostability of piroxicam after irradiation

Time hours	Content of piroxicam %
o	99.8
24	99.7
48	99.6
72	99.6

7. Drug Metabolic Products, Pharmacokinetics, Bioavailability

Piroxicam's pharmacological and pharmacokinetic profile,has been rationalised in terms of chemical groupings

SCHEME 3.



of the molecule. The enolic portion has a relatively low pK_a which leads to virtually complete ionizations and prolongs the activity of the drug. The group is also necessary for prostaglandin biosynthetase inhibition, the proposed mode of action of piroxicam. The sulphoxide group is lipophilic, enhancing absorption and facilitating the passage of piroxicam across the gut-blood barrier. The heterocyclic side-chain increases acidity and lipophilicity, it slows hydroxylation and therefore prolongs the half-life. The N-methyl group increases efficacy by a greater inhibition of PG synthetase than that provided by a larger chain.

7.1. Pharmacokinetics

Piroxicam is readily absorbed after oral or rectal administration and accumulates after repeated doses to reach steady-state after about 7 days. The drug is extensively metabolised to apparently inactive metabolites and has a half-life of about 4o hours in man.

Peak plasma concentrations are attained about 2 hours after a single oral dose and at about 5.5 hours after rectal administration as suppositories /21/. Due to the extended plasma half-life of piroxicam, plasma concentrations remain very stable over the next 24-48 hr. Mean peak plasma concentrations are roughly related to dosage between lo and loo mg, being o.85 μg/ml and 13.5 μg/ml after a single lo or loo mg dose, respectively /22/. At concentrations of between 5 and 3o μg/ml, piroxicam is 99.3% bound to plasma proteins /21,22/. Piroxicam penetrates into the synovial fluid of patients with rheumatoid arthritis and attains concentrations of about 4o% of those in plasma.

Piroxicam is highly protein-bound and thus might be expected to displace other protein-bound drugs. Its absorption and disposition are unaffected by concomitant administration of aspirin and antacids.

7.2. Metabolism

Available data suggest that piroxicam is extensively metabolised in man /23/ by the routes of 5'-pyridine ring hydroxylation, glucuronide formation, cyclodehydration and amide hydrolysis leading to decarboxylation, ring contraction and N-dealkylation /24/. The metabolic pathways recognized for piroxicam are shown in Scheme 3. The principal metabolite in man is that produced by hydroxylation of the pyridyl ring (metabolite B) and exists either free or conjugated with glucuronic acid. The metabolite is at least looo times less active than piroxicam in inhibiting

prostaglandin synthetase /25/.

About 10% of a single oral dose of piroxicam is recovered unchanged in the urine within the first 8 days after administration. The elimination of half-life of piroxicam is extended due to a low clearance rate and has most often been calculated at 38 hours range 31 to 56.7 hours in healthy subjects /21-26/. Half-life appears not to be related to dose or plasma concentration.

The formation of the cyclodehydrated metabolite (A) is a major pathway in the dog, while the rat seems to hydroxylate the pyridyl ring in the para-position to form metabolite B. The latter compound, either free or conjugated with glucuronic acid, is the major human metabolite, accounting for up to 60% of a daily dose in urine and feces. Laboratory animals also deamidate, decarboxylate, and cause ring contraction, all in varying amounts. These latter processes seem to be of minor importance in man /27/.

None of the metabolites of piroxicam have significant antiinflammatory activity in laboratory animals /28/. The lack of activity for the metabolites, together with their very low concentration in plasma (at or below the limits of analytical detection), leads to the hypothesis that piroxicam is intrinsically active, and its metabolites do not contribute to that activity. Apparently, piroxicam embodies a unique combination of functional groups which are all required for its action.

8. Toxicity

Acute toxicity of piroxicam is low: the LD_{50} for orally applied piroxicam is 360 mg/kg in the mouse, 270 mg/kg in the rat and over 700 mg/kg in the dog. When administered intraperitoneally, the LD_{50} values are 360 and 220 mg/kg in the mouse and rat, respectively /6,29/.

9. Identification and Determination in Body Fluids and Tissues

Piroxicam in plasma was assayed spectrophotometrically at 355 nm. The sensitivity limit of the assay was approximately 0.5 μg/ml /22/. As well, piroxicam in plasma was assayed fluorometrically after hydrolysis with 6N H_2SO_4 for 20 hours at 105°C. The released 2-amino-pyridine was examined in an Aminco Bowman spectrofluorimeter with excitation at 310 nm and measurement at 380 nm. A Gilford Model 410 digital absorbance meter and a Gilford Model 4006 data lister were interfaced with the spectrofluorimeter for recording of the fluorescence values. The assay was linear and sensitive down to 0.5 μg/ml. High concen-

trations of salicylate were found not to interfere in the assay of piroxicam /22/.

1o. Identification and Determination in Pharmaceuticals

Piroxicam in capsules was determined spectrophotometrically by measuring the extinction of the solution at 34o nm, 1 mg/1oo ml, against a suitable blank, and the content of piroxicam in 1 capsule was calculated /15/.

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RANITIDINE

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Nikola Blažević

6. Stability - Degradation
7. Drug Metabolism, Pharmacokinetics, Bioavailability
8. Identification and Determination in Body Fluids and Tissues
9. Identification and Determination in Pharmaceuticals
1o. References

1. Foreword, History, Therapeutic Category

Ranitidine is a new histamine H_2-receptor antagonist which, unlike cimetidine that contains an imidazole ring, has a furane ring structure /1,2/. This substituted amino--alkyl-furane derivative is more potent than cimetidine in inhibition of gastric acid secretion induced by various stimuli and lacks cimetidine's anti-androgenic and hepatic microsomal enzyme inhibiting effects.

The drug has been used in the treatment of duodenal and gastric ulceration. In the recommended dosage of 150 mg twice daily, ranitidine is as effective as cimetidine and has therefore the advantages of less frequent dosing and fewer side effects. Ranitidine appears to be the drug of choice in the treatment of the Zollinger-Ellison syndrome because of its increased potency and lesser effect on endocrine function compared to cimetidine. The compound is given orally as a tablet (150 mg of ranitidine base) and as an injection solution (50 mg/5 ml).

The first synthesis of ranitidine was reported in 1973 /3/ followed by pharmacological and clinical studies in 1979 /1,4/ and 1980 /5-7/. Finally, ranitidine was introduced on to the market in 1981.

2. Description

2.1. Nomenclature

2.1.1. Chemical Name

The Chemical Abstracts name for ranitidine is N-[2--[[[5-[(dimethylamino)methyl]-2-furanyl]methyl]thio]ethyl]--N'-methyl-2-nitro-1,1-ethene diamine. The CAS registry No. is 66357-35-5. The other name is 2-[[[5-(dimethylamino)-methyl-2-furanyl]-methyl]thio]-ethylamino-2-methylamino--1-nitroethene.

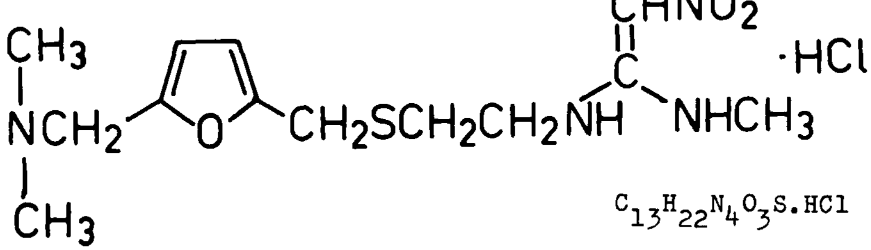
2.1.2. Generic Name

Ranitidine

2.1.3. Trade Name

Zantac

2.2. Formula



$C_{13}H_{22}N_4O_3S.HCl$

2.3. Molecular Weight

350.869

2.4. Appearance, Color, Odor

Ranitidine is marketed only as the hydrochloride salt. It is a white to yellowish solid with little or no odor. A slight sulfur-mercaptan odor may be present.

3. Synthesis

The first synthesis of ranitidine, as shown in Scheme I, started with the reaction of 5-dimethylaminomethyl-2-furanyl-methanol (I) with 2-mercaptoethylamine by means of aqueous hydrochloric acid to give 2-[[(5-dimethylaminomethyl-2-furanyl)methyl]thio]ethaneamine (II). This intermediate is then condensed with N-methyl-1-methylthio-2-nitro etheneamine (IV) by heating in water at 50°C for 4 hours. Compound IV is obtained in the reaction of 1,1-bis(methylthio)-2-nitroethene (III) with methylamine in refluxing ethanol /3/.

Alternatively, ranitidine (V) may be prepared by condensation of 5-dimethylaminomethyl-2-furanyl methyl mercaptan (VI) with aziridine derivative VII /8/ and by the reaction of amine II with 1-nitro-3-methyl ketene imine (IX) /9/ - Scheme II. Other patented methods for ranitidine preparation are also shown in Scheme II. The reaction of nitromethane with pseudothioureido compound VIII /10/ and carbodiimide XI /11/ as well as the condensation of aziridine with nitroethene intermediate X /12/ and mercapto diamino nitroethene XII with 5-dimethyl-aminomethyl-2-furanylmethanol (I) /3/ leads to ranitidine in moderate yields.

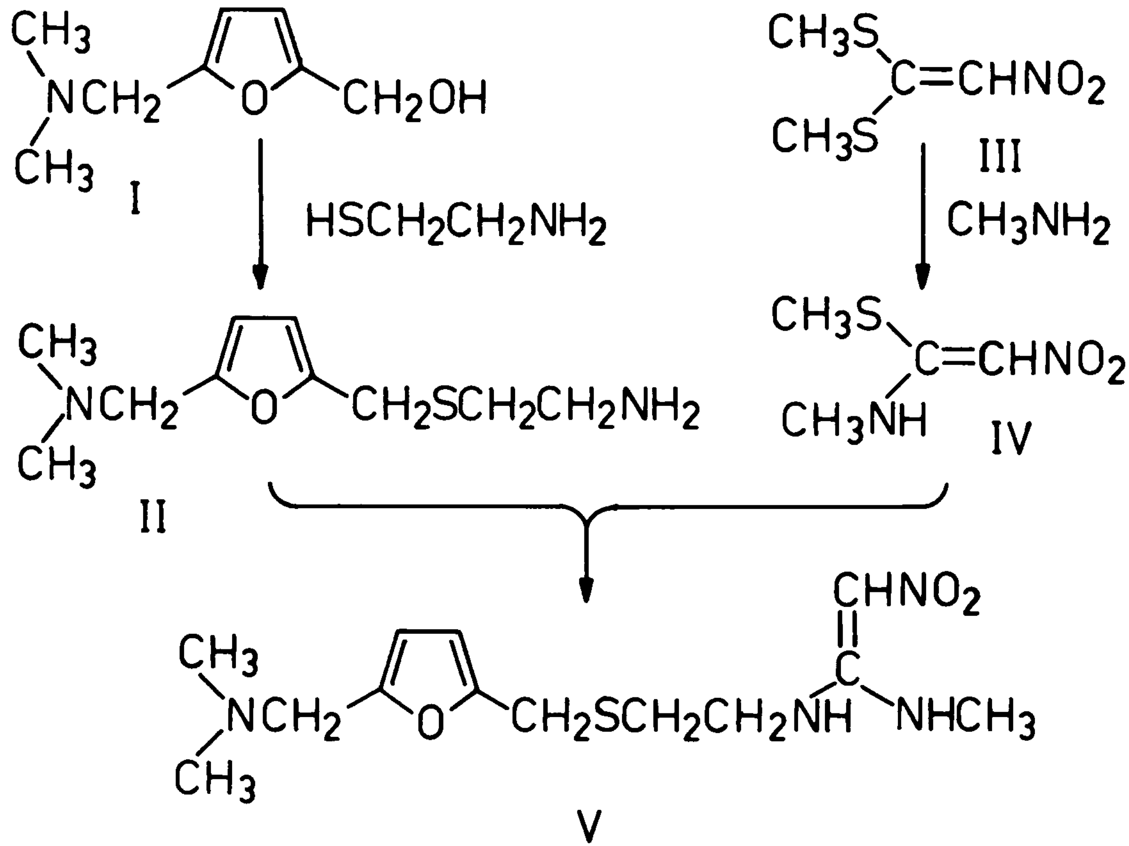
4. Physical Properties

4.1. Spectral Properties

4.1.1. Infrared Spectra

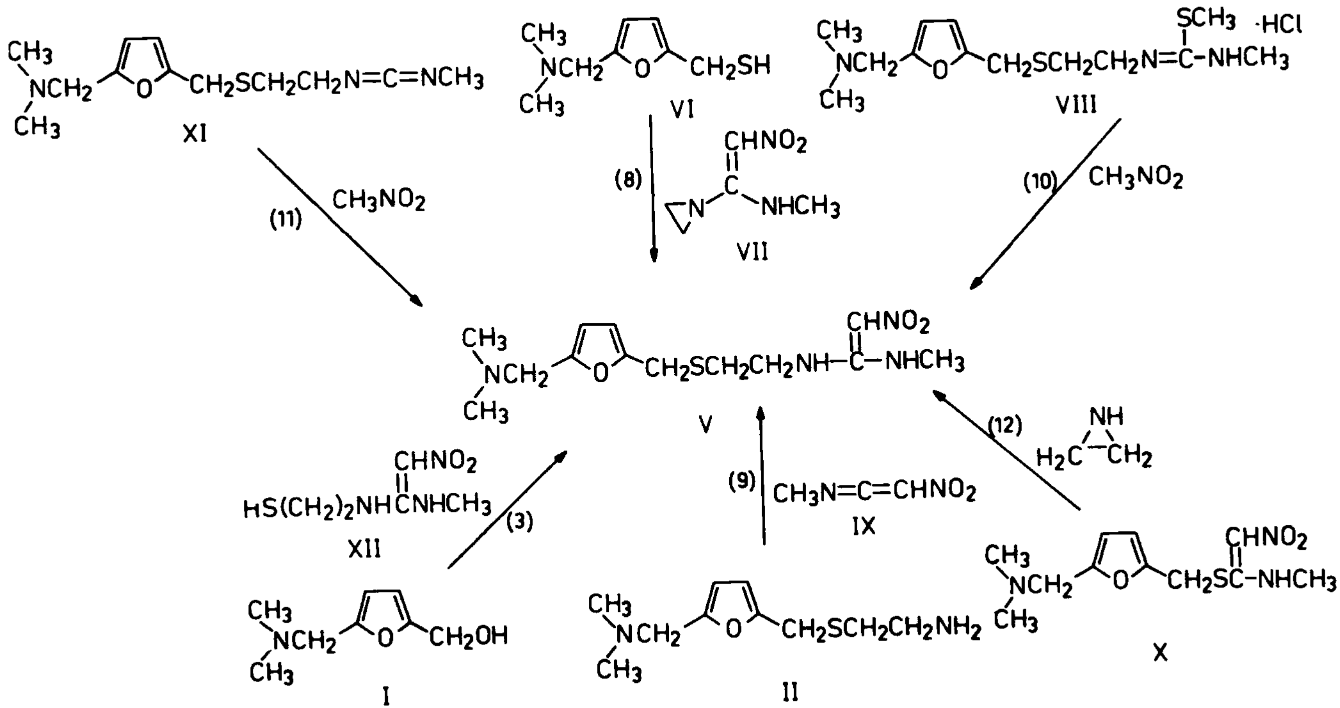
The IR absorption spectrum of the crystal form 1 ranitidine hydrochloride is shown in Figure 1. Figure 2 is the infrared spectrum of the form 2 ranitidine hydrochloride. These spectra were recorded with KBr-pelleted samples using a Pye Unicam SP-200 Infrared Spectrophotometer /13/. Our results are in good agreement with the published data for the crystal form 2 ranitidine hydrochloride /14/. In the IR spectrum of the crystal form 1 (Fig. 1) the well-known bands characteristics of a nitro group attached to a saturated carbon atom i.e. stretching vibration frequencies at 1554 and 1382 cm^{-1} /15/, are hardly visible as well as the bands in the range of 1505-1540 cm^{-1} characteristic of a nitro group attached to a substi-

SCHEME I.



CH3
NCH2
O
CH2OH
CH3
I
HSCH2CH2NH2
CH3S
C=CHNO2
CH3S
III
CH3NH2
CH3
NCH2
O
CH2SCH2CH2NH2
CH3
II
CH3S
C=CHNO2
CH3NH
IV
CHNO2
C
CH3
NCH2
O
CH2SCH2CH2NH
NHCH3
CH3
V

SCHEME II


XI
VI
VIII
VII
V
XII
IX
X
I
II
(11)
CH_3NO_2
(8)
(10)
(3)
(9)
(12)
$CH_3N{=}C{=}CHNO_2$
$HS(CH_2)_2NHC(=CHNO_2)NHCH_3$

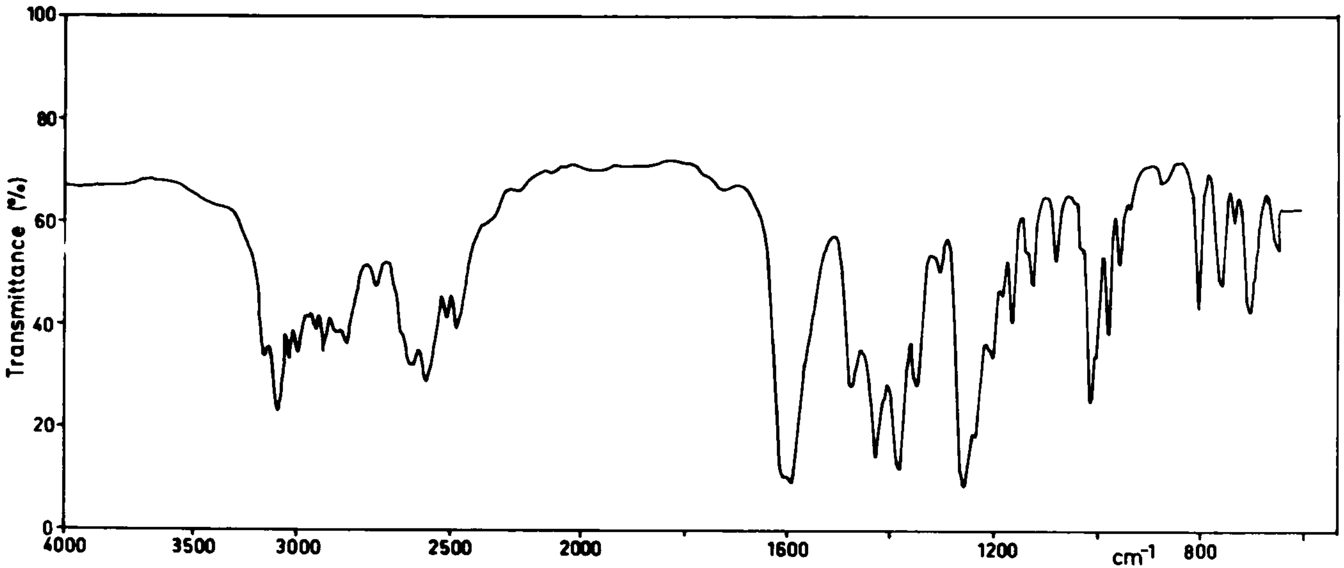


Fig. 1. Infrared spectrum of ranitidine hydrochloride form 1 in KBr pellet.
Instrument: Pye-Unicam SP3-200.

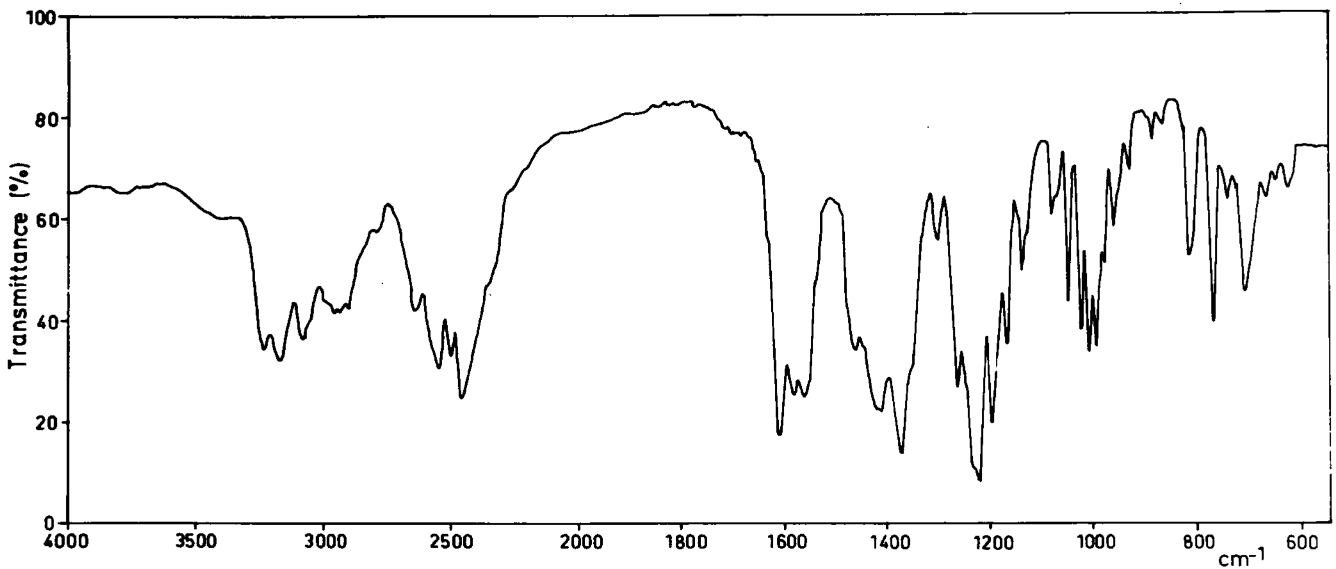
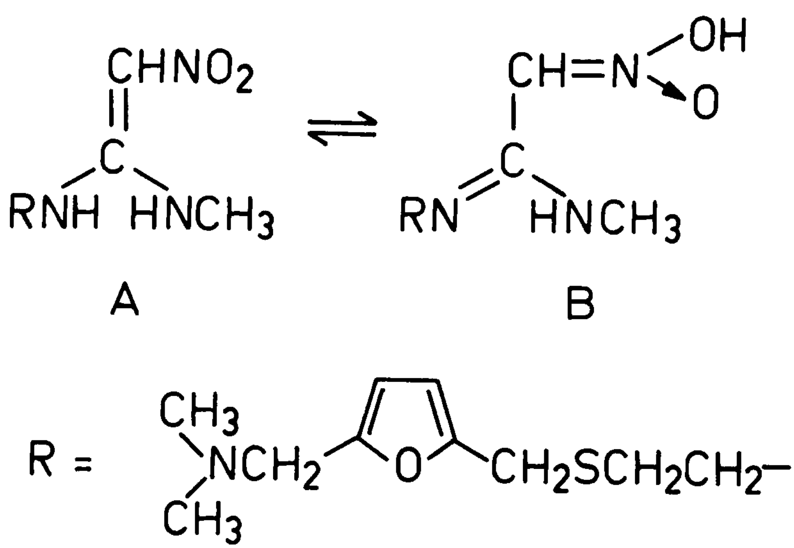


Fig. 2. Infrared spectrum of ranitidine hydrochloride form 2 in KBr pellet.
Instrument: Pye-Unicam SP3-2oo.

tuted ethene group /16/. There is, however, a strong band at 1620 cm^{-1} which corresponds to the stretching vibration of the C=N double bond in an aci-nitro group of nitronic acid /17/. Markedly strong bands appearing at 2640 and 2560 cm^{-1} are characteristic of the N^+-H bond which exists in the protonated tertiary amine group $H-N^+R_3$ in V hydrochloride. The properties of the IR spectrum suggest that ranitidine hydrochloride exists mostly in the tautomeric form denoted by B in Scheme III. This conclusion is in agreement with other authors' findings for similarly substituted nitroethenes in which nitronic acid forms are stabilized by conjugation /18/. Other spectroscopic data also suggest that ranitidine hydrochloride exists predominantly in the form represented by formula B. These conclusions were also confirmed by single crystal X-ray diffraction studies reported by B. Kojić-Prodić et al /19/. Namely, the bond lengths in the 2-ethyl-2-methylamino-1-nitroethene residue are in agreement with the structure B.

SCHEME III



4.1.2. Ultraviolet Spectrum

The ultraviolet spectrum of ranitidine hydrochloride was recorded in an aqueous solution, concentrations 10^{-2} g/l, using a Pye Unicam SP8-100 UV-spectrophotometer, and is shown in Figure 3 /13/. In the ultraviolet spectrum of V hydrochloride an expected bathochromic shift of the absorption maximum, characteristic for nitro group - olefin

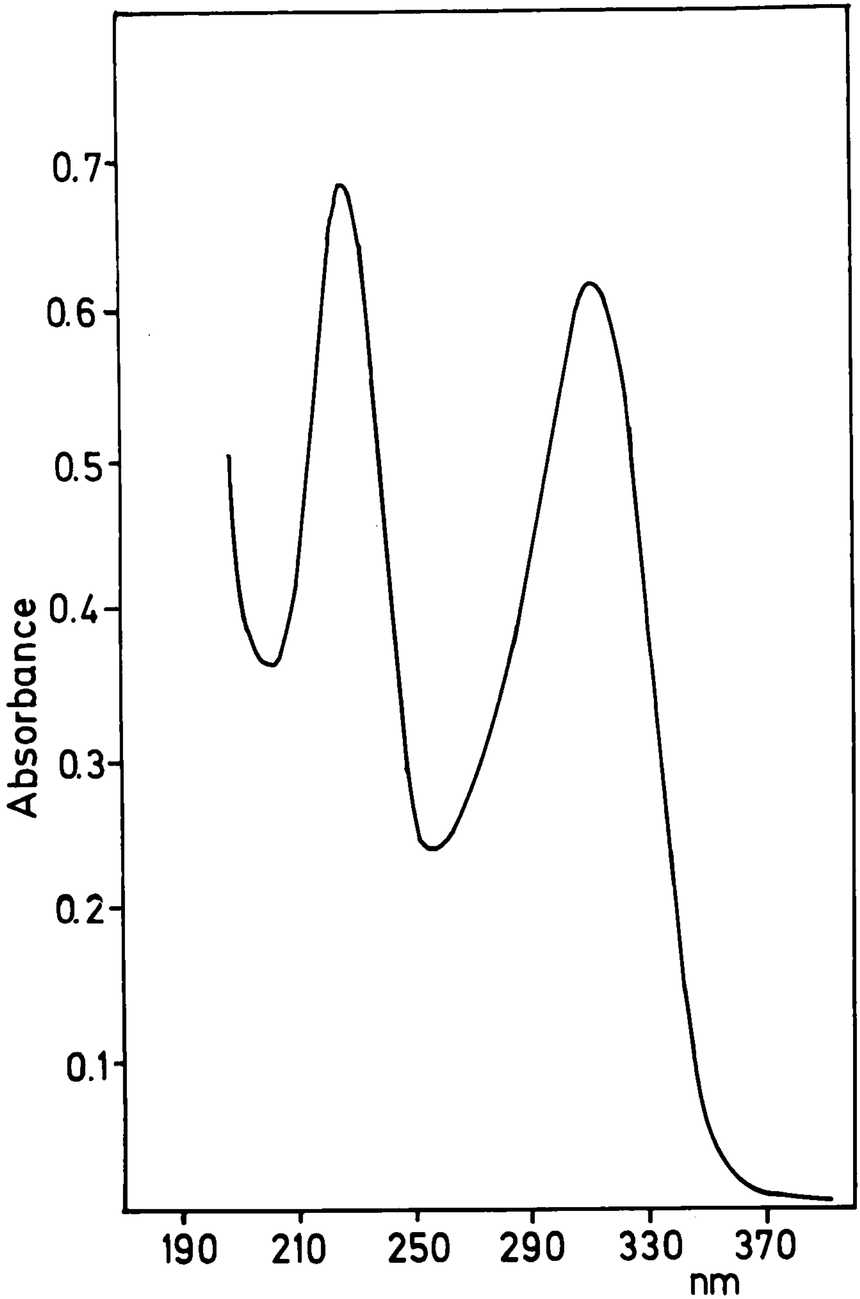


Fig. 3. Ultraviolet spectrum of ranitidine hydrochloride in an aqueous solution. Instrument: Pye-Unicam SP8-1oo.

conjugation, is present. The spectrum shows two absorption maxima, at 228 nm (ε=23.485) and at 313 nm (ε= =16.030). The measurement of the absorption at 313 nm is very convenient for quantitative determination of ranitidine hydrochloride even in the presence of intermediates in the ranitidine synthesis.

4.1.3. Proton Magnetic Resonance

The proton magnetic resonance spectra of ranitidine base (Figure 4) and ranitidine hydrochloride (Figure 5) were recorded with a WP 80PS instrument by Bruker /13/. The spectra illustrated were obtained using deuterated chloroform and deuterated dimethyl sulfoxide solutions containing approximately 80 mg/ml of the compound with tetramethylsilane as the internal standard. The curve denoted by B in both spectra represents the case when D_2O was added to deuterated organic solvent solutions. The spectrum of ranitidine base shows a singlet at 2.24 ppm (due to six protons of the $N(CH_3)_2$ group) and a broad doublet at 3.00 ppm (due to protons of the CH_3NH group) which, upon addition of D_2O, contracts into a singlet. Spin decoupling at 3.40 ppm - the position of a multiplet due to the methylene protons of the $NHCH_2$ group - causes a contraction of the upfield triplet at 2.84 ppm to a singlet. As the coupling constant J(CH_2S,CH_2N) is 7.5 Hz, the triplet at 2.84 ppm sensitive to decoupling influences must correspond to the two protons of the methylene group connected with the sulfur atom, which points away from the ring. Further signals may be assigned as follows: the singlet at 3.50 ppm (2H) corresponds to protons of the CH_2N group in 5-position of the furane ring. The next downfield singlet at 3.72 ppm (2H) corresponds to the protons from the methylene bridge linking the ring to the S-atom. The pair of doublets at 6.22 ppm (1H) and 6.26 ppm (1H) corresponds to ring protons in 3- and 4-positions, respectively ($J_{3,4}$=3.75 Hz). The nearest singlet positioned at 6.56 ppm (1H) corresponds to the vinyl proton of the nitroethene group $CHNO_2$. In the spectrum of ranitidine hydrochloride (Figure 5) the signal for this proton is situated between signals for the furane ring protons. This proton is in both cases displaced by a deuteron upon addition of D_2O, which indicates a tautomeric prototropic shift in the nitroethene terminal. This observation has been confirmed by A. Sega, et al. /20/ in their investigation of the H/D exchange in 2,2-disubstituted nitroethenes. Finally, the broad singlet centered at 10.33 ppm (1H) cannot be definitely assigned to either the proton NH, or that of the nitronic acid group =N-OH. The contrac-

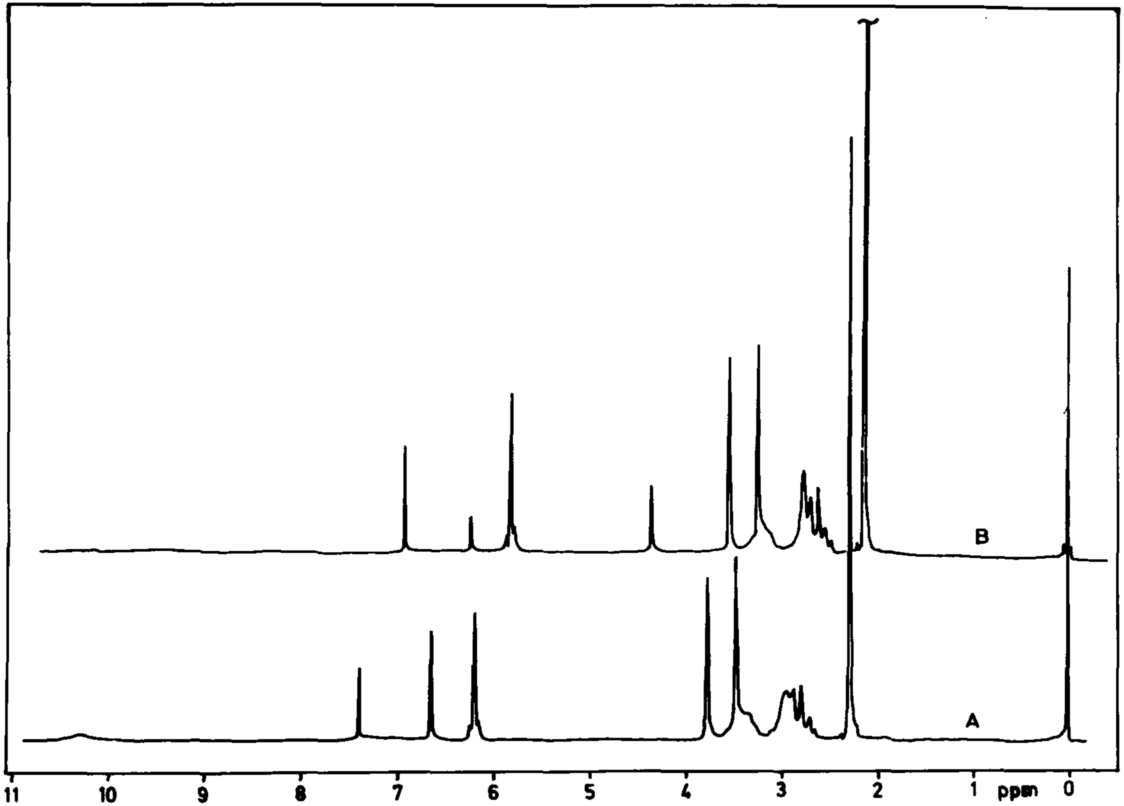


Fig. 4. Proton magnetic resonance spectrum of ranitidine base in $CDCl_3$.
Instrument: Bruker WP8oPS

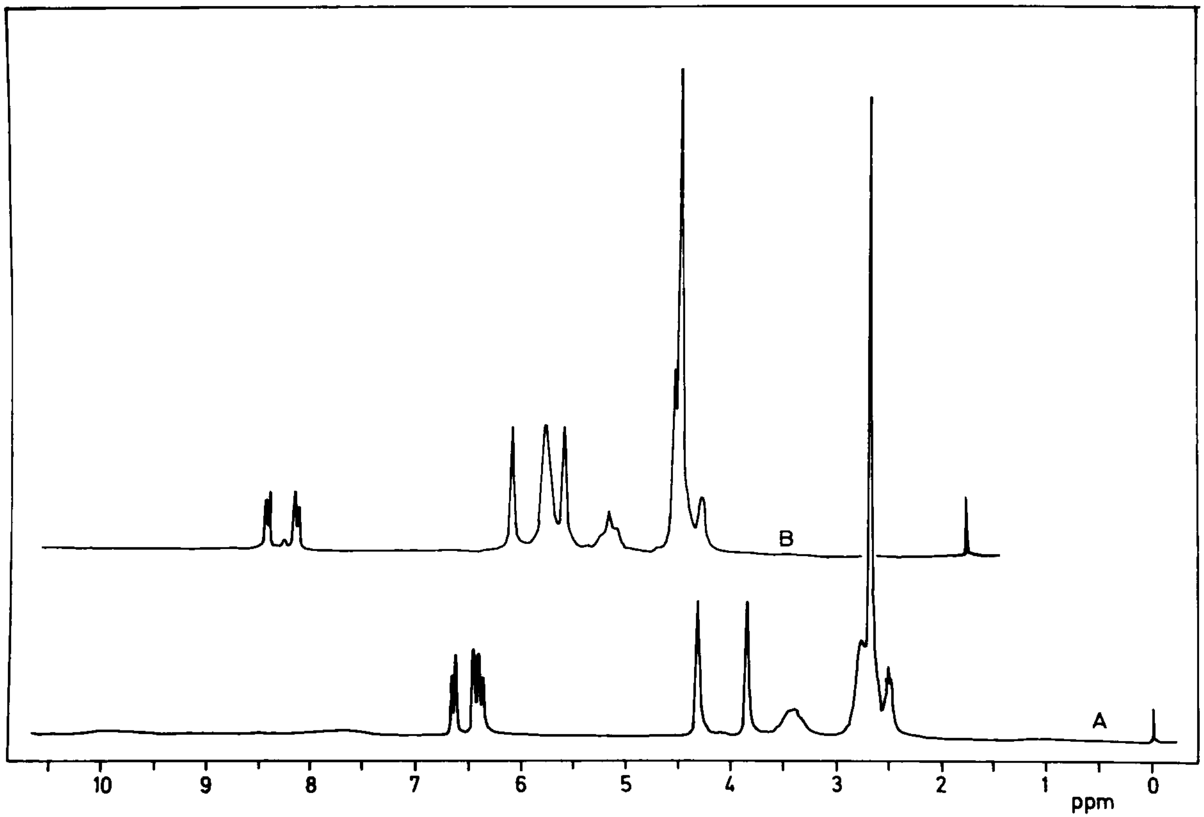


Fig. 5. Proton magnetic resonance spectrum of ranitidine hydrochloride in DMSO-d_6.
Instrument: Bruker WP8oPS.

ting effect of D_2O addition upon the CH_3NH- multiplet confirms that the neighbouring N-atom binds a proton. The influence of D_2O does not extend to the CH_2N= multiplet which confirms the absence of a proton from N-atom. One should expect, therefore, that ranitidine is more likely to be represented by formula B (Scheme III) than by formula A.

4.1.4. ^{13}C-Nuclear Magnetic Resonance

The ^{13}C-NMR spectra of ranitidine hydrochloride were recorded with a Jeol FX-1oo spectrometer at 25.o5 MHz. The samples were dissolved in DMSO-d_6 and spectra were recorded in broad-band, off-resonance and NOE modes /13/. The broad-band and off-resonance spectra were shown in Fig. 6. In both modes it was possible to assign all carbon atoms. The determinations of C-H coupling constants were difficult for groups $N(CH_3)_2$ and $NHCH_3$, because the respective signals were situated within the range of the septet corresponding to the solvent DMSO-d_6 . Therefore, they were determined using NOE measurements in CD_3OD at 2o.1 MHz.

Table I. ^{13}C-NMR spectrum of ranitidine hydrochloride in CD_3OD at 2o.1 MHz; chemical shifts (δ) and C-H coupling constants (J_{C-H})

C-atom	δ (ppm)	J_{C-H} (Hz)
N≷C- HN	158.o doublet	6.3
C-5 furane	155.o doublet	12.6
C-2 furane	145.o doublet	12.6
C-4 furane	116.6 quartet	128.1 and 5.o
C-3 furane	11o.2 quartet	123.9 and 5.o
$CH=NO_2H$	99.3 doublet	189.2
$N-CH_2$	53.8 triplet	148.3
$N(CH_3)_2$	42.8 quartet	126.3
$NHCH_3$	42.3 quartet	139.1
$-CH_2N-$	31.7 triplet	15o.1
$-SCH_2-$	28.9 triplet	142.2
$-CH_2S-$	28.6 triplet	139.1

4.1.5. Mass Spectrum

The mass spectrum was recorded with an MS-25 mass spectrometer with data system DS 5oS from Kratos, Manchester. Samples for mass spectra were directly introduced into the ion source. The electron impact ionisation was applied at 7o eV (473 K source temperature) and spectra were recorded at a scanning speed of 3 sec/scan. The spe-

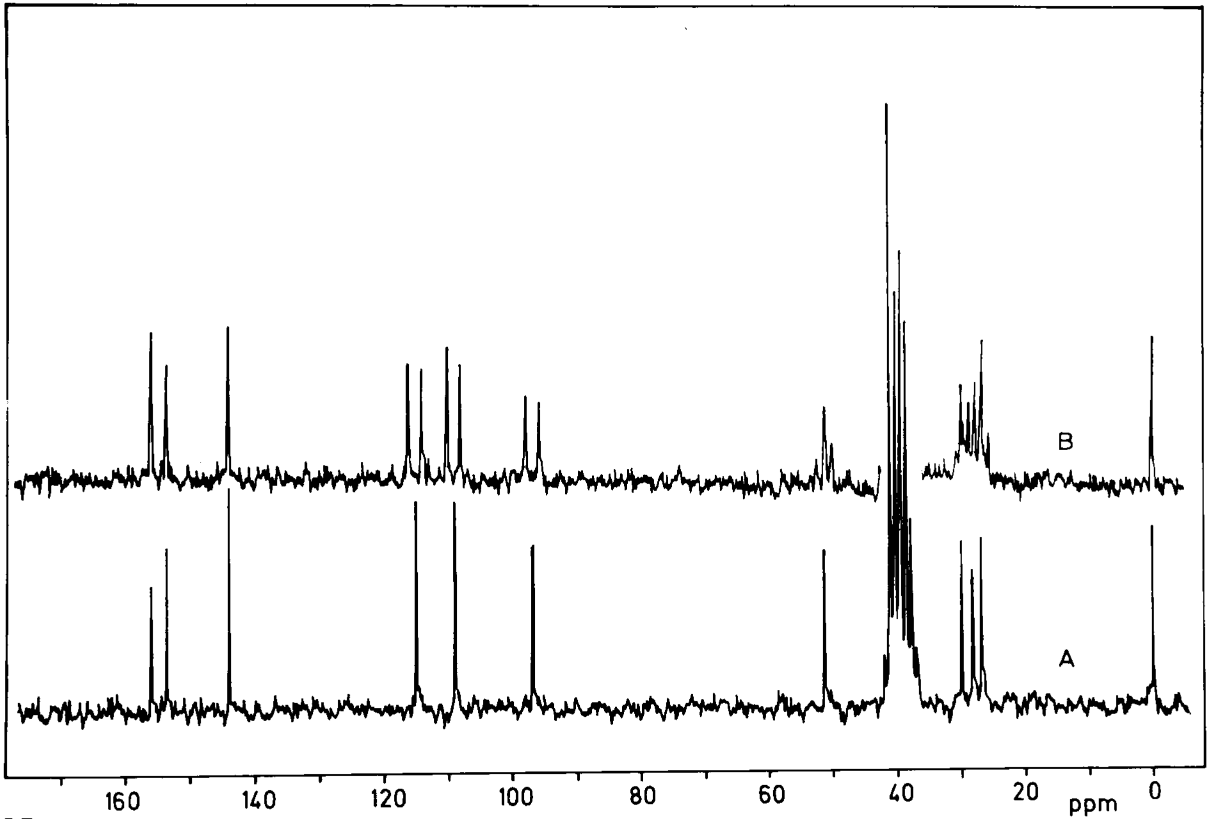


Fig. 6. ^{13}C-NMR broad-band (A) and off-resonance (B) spectra of ranitidine hydrochloride in DMSO-d_6. Instrument: Jeol FX-100 at 25.05 MHz.

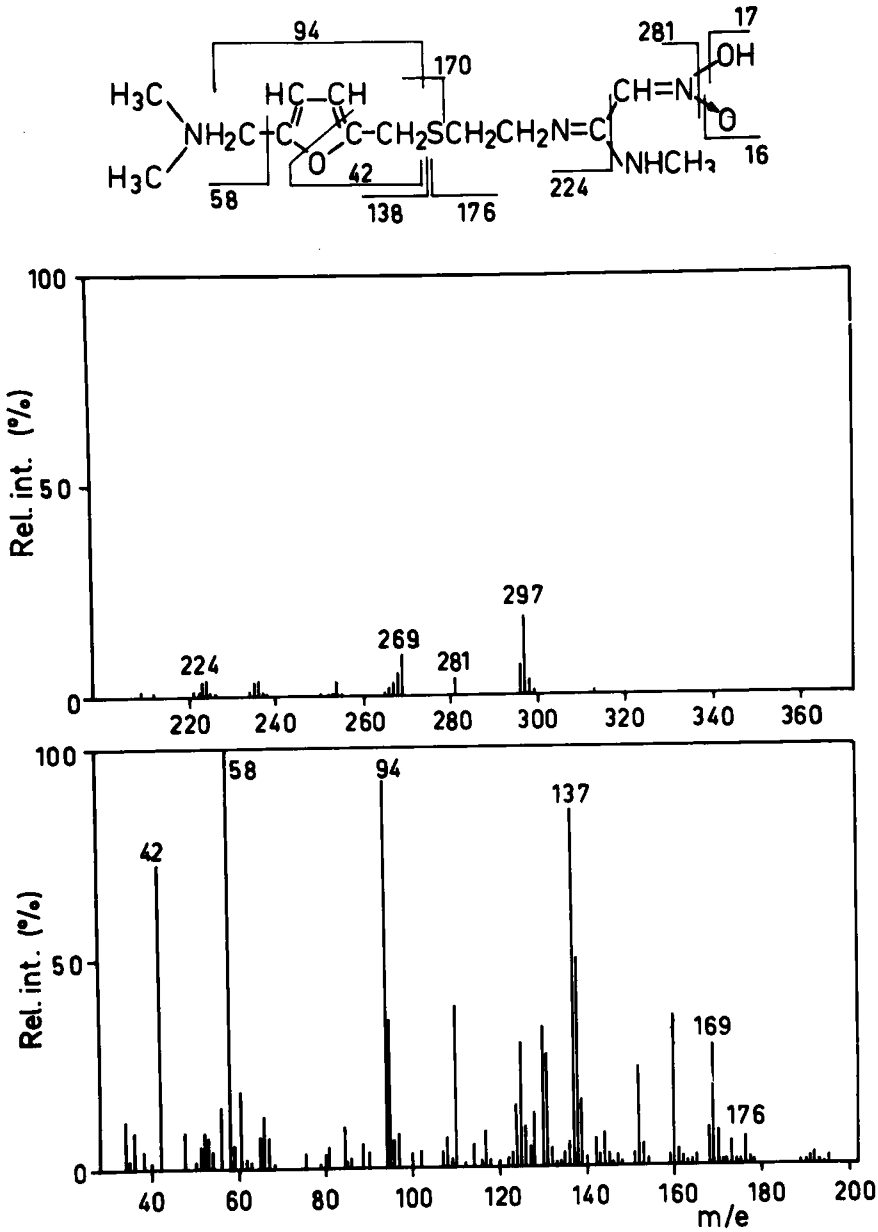


Fig. 7. Mass spectrum and fragmentation of ranitidine. Instrument: Kratos MS-25.

Table II. X-Ray diffraction data of ranitidine hydrochloride form 1

Θ (°)	Interplanar distance d* (Å)	Relative intensity** I/I_o
7.02	6.31	22
7.21	6.14	34
7.59	5.84	39
7.76	5.71	79
8.43	5.26	100
8.66	5.12	34
10.36	4.29	21
10.81	4.11	87
11.16	3.98	64
11.97	3.72	24
11.34	3.61	76
12.12	3.40	59
13.07	3.17	73
14.24	3.13	45
14.49	3.08	42
14.18	2.04	21

*$d = n\lambda / 2 \sin\Theta$

**Based on the highest intensity adjusted to 1.00.

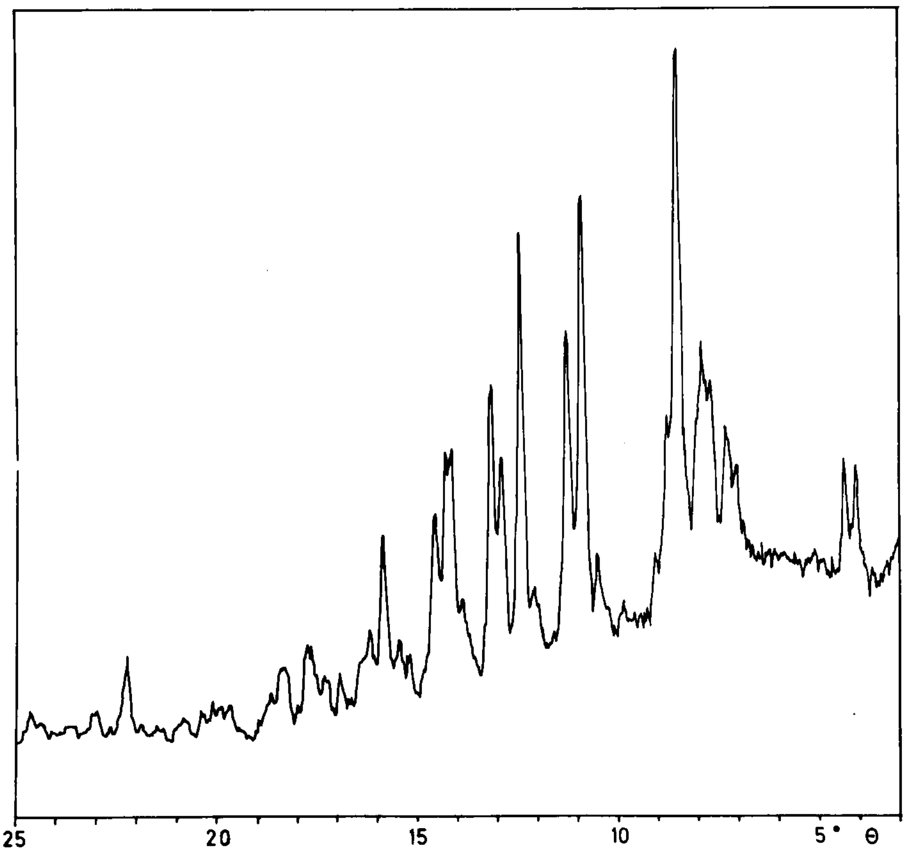


Fig. 8. X-Ray diffraction pattern of ranitidine hydrochloride form 1. Instrument: General Electric XRD-6 spectrogoniometer.

Table III. X-Ray diffraction data of ranitidine hydrochloride form 2

Θ (°)	Interplanar distance d* (Å)	Relative intensity** I/I_o
4.2	10.47	20
7.3	6.08	13
7.7	5.75	21
8.3	5.34	37
9.1	4.87	12
9.6	4.62	11
10.2	4.36	100
10.5	4.23	15
11.4	3.88	14
11.8	3.77	58
12.1	3.67	23
12.4	3.60	12
12.9	3.44	15
13.8	3.23	21
14.4	3.10	19
14.8	3.02	7
16.0	2.79	20
18.2	2.46	9

* $d = n\lambda/2 \sin\Theta$

** Based on the highest intensity adjusted to 1.00.

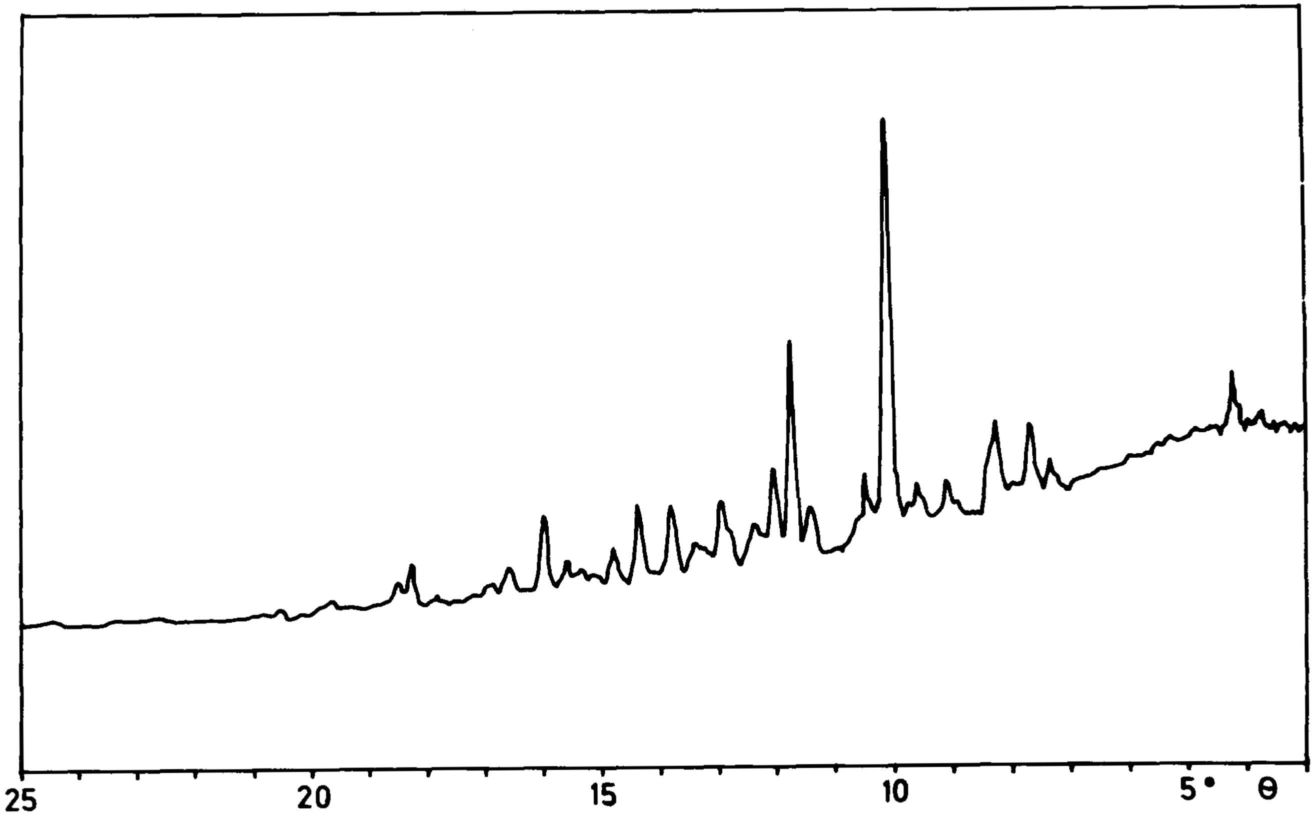


Fig. 9. X-Ray diffraction pattern of ranitidine hydrochloride form 2.
Instrument: General Electric XRD-6 spectrogoniometer.

ctra were evaluated using the attached data system /13/. The mass spectrum of ranitidine and its main fragments are shown in Figure 7. The molecular ion on the ranitidine spectrum could not be detected. The abundance of the ion m/e 297, which differs by 17 mass units from the proposed molecular ion, suggests the presence of a hydroxyl group in the structure of ranitidine. Loss of OH from the molecular ion was confirmed by exact mass measurements of the m/e 297 by high resolution mass spectrometry. This fragmentation is in accordance with our proposal that the structure of ranitidine is described by formula B (Scheme III). According to Martin, et al. /21/ the ion m/e 297 was generated by loss a molecule of water from the protonated molecular ion. It should be noted that this mass spectrum was obtained by the chemical ionization mode, while we used an electron impact technique.

4.2. Solid Properties

4.2.1. Melting Characteristics

The ranitidine base is difficult to crystallize, but its hydrochloride can be conveniently crystallized, particularly from isopropanol. The melting range of ranitidine hydrochloride depends on the polymorphic form in which this compound is crystallized. When ethylacetate is added to an ethanolic solution of ranitidine hydrochloride the crystalline form 1 is obtained with the melting range of 135-136°C /ref. 4: m.p. 133-134°C/. The form 2 is crystallized from isopropanol-HCl solution with the melting range of 143-144°C /ref. 14: m.p. 141-142°C/.

4.2.2. X-Ray Diffraction

The X-ray diffraction patterns were determined with a Mod. XRD-6 spectrogoniometer from General Electric, Schenectady. The spectra were taken with a monochromatic radiation which was obtained from the CuK_{α} line (15.42 nm) excited at 35 kV and 2o mA /13/. The X-ray powder diffraction spectra for both forms of ranitidine hydrochloride are given in Figures 8 and 9. Tables II and III list the interplanar distances, the diffraction angle and the relative peak intensities.

4.3. Solution Properties

4.3.1. Solubility

The solubility of ranitidine hydrochloride in various solvents at room temperature is summarized in Table IV.

4.3.2. Acidity (pK_a)

For the determination of the pK_a value the spectro-

Table IV. Solubilities of ranitidine hydrochloride

Solvent	Solubility
acetic acid	freely soluble
water	very freely soluble
methanol	soluble
ethanol	sparingly soluble
ethylacetate	very slightly soluble
isopropanol	very slightly soluble
dioxane	insoluble
chloroform	insoluble

photometric method was used /22/. The obtained value was 2.19 ± 0.04 /13/.

5. Methods of Analysis

5.1. Chromatographic Methods

5.1.1. Thin Layer

The purity of ranitidine hydrochloride can be quickly assessed by TLC over silica gel. Table V shows its R_f-values with several solvent systems /13/. Spots were located either under an UV lamp, or by staining through exposure to iodine vapors.

Table V. R_f-values

Solvents and R_f-values					
A	B	C	D	E	F
0.50	0.44	0.36	0.64	0.73	0.39

A= EtOAc/MeOH/Et_2NH (3:3:1), B= $CHCl_3$/i-PrOH/Et_2NH (4:3:2), C= dioxane/MeOH/DMF (6:3:2), D= MeCN/MeOH/25% NH_4OH (5:2:1), E= EtOAc/MeOH/25% NH_4OH (1:5:1), F= EtOAc/i-PrOH/25% NH_4OH (4:3:1).

Suggested procedure for the identification of ranitidine by thin-layer chromatography: five, 15 and 30 μl samples of a 20 mg.cm^{-3} methanolic solution, and the same volume of a methanolic solution of equal concentrations of the standard substance are applied to a silica gel plate and chromatograms developed with ethylacetate/methanol/diethylamine (3:3:1). The spots must have the same fluorescence intensity under UV-radiation, and the same shade of strain subsequent to exposure to iodine vapor. All R_f values must be close to 0.5.

5.1.2. High Pressure Liquid

The HPLC analyses were performed with a HPLC apparatus, LC-3-XP with an UV-LC detector. The HPL chromatogram is shown in Figure 1o /13/. The chromatograms were run through a column filled with Li-Chromosorb RP-8 (5 μm) using a mixture of acetonitrile, methanol, water, and concentrated ammonia (25o, 2o, 6 and o.o5 cm^3, respectively) having a pH-value of 7.4. The elution was carried out under 13.78 to 17.22 MPa (2o.ooo-25.ooo p.s.i.) pressure, maintaining a flow rate of 1 $cm^3.min^{-1}$. The effluent was monitored optically at 217 nm.

5.2. Spectrophotometric Determination

Ten mg of "unknown" ranitidine hydrochloride is accurately weighed into a 1oo cm^3 volumetric flask, dissolved by swirling with 5o cm^3 of water and the solution made up to the mark. Ten cm^3 of the resulting solution is diluted to 1oo cm^3 in another volumetric flask, and the absorbance of the final dilution is measured at 313 nm against water. The same procedure is carried out with a standard sample of known ranitidine concentration /13/.

Calculation:

$$C_{13}H_{23}ClN_4O_3S \text{ in unknown, } \% = \frac{A_u M_s P}{A_s M_u} \cdot 1oo$$

A = absorbances, M = masses (g), u = unknown, s = standard, P = % of $C_{13}H_{23}ClN_4O_3S$ in the standard.

6. Stability - Degradation

The stability of ranitidine hydrochloride in tablets was tested in two ways: one series of samples was kept at 4o °C and 5o-6o% relative humidity for five days, and the other at 6o °C and 1oo% relative humidity during the same period. According to the spectrophotometric determination, the degradation amounted to ~5% (under the first conditions) and 11.5%, respectively.

The thin-layer chromatographic analysis on silica gel shows five different degradation products in both cases.

7. Drug Metabolism, Pharmacokinetics, Bioavailability

Metabolic studies of ^{14}C-ranitidine in rat and dog showed that ranitidine was mainly metabolised by oxidation to give N-oxide XIII, S-oxide XV, and desmethyl ranitidine XIV. The relative amount of each metabolite formed was found to vary with the species /23/. Thin-layer chromatographic analyses of the urine collected from volunteers given oral and intravenous doses of ranitidine

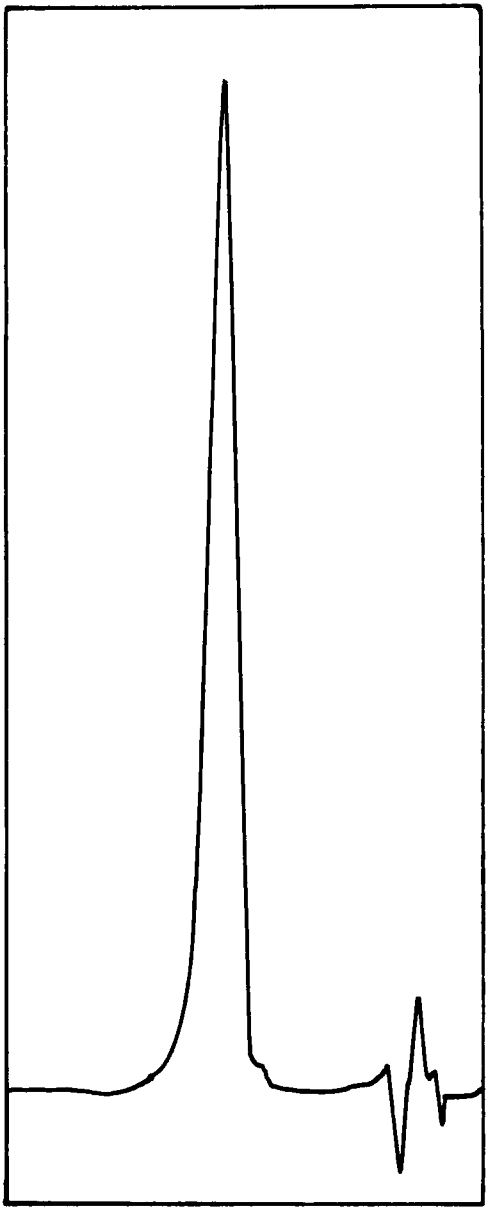
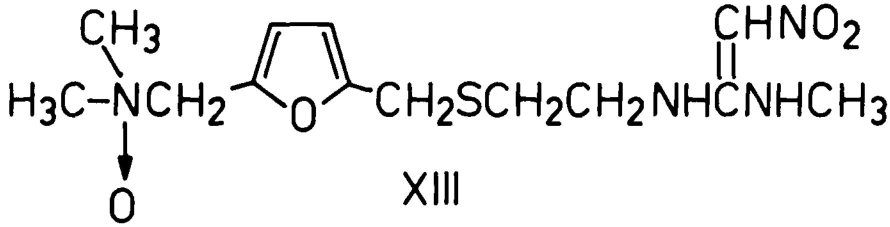
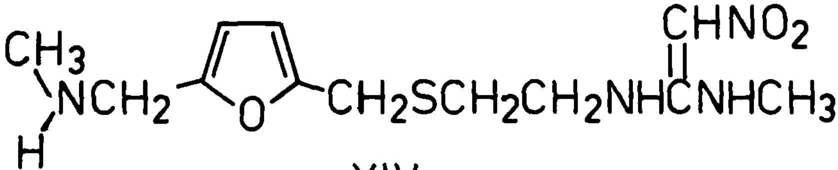


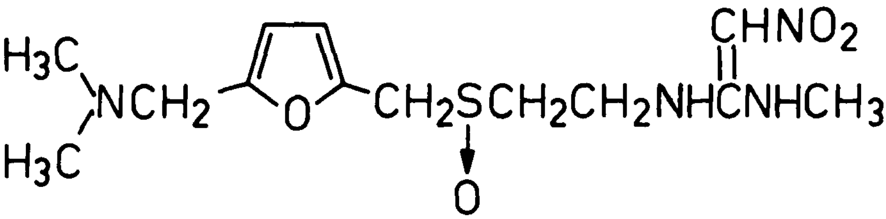
Fig. 1o. HPL chromatogram of ranitidine hydrochloride.
Instrument: Pye-Unicam LC-3-XP.



XIII



XIV



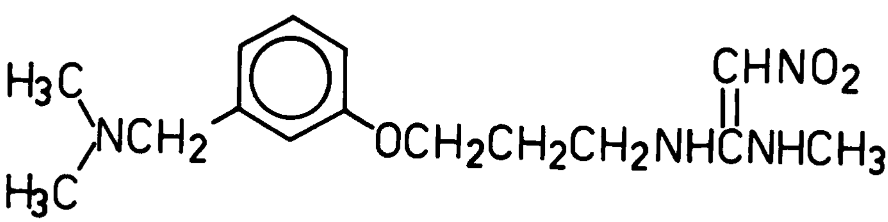
XV

showed that ranitidine was the major component present. Compound XIII was the major metabolite, and small quantities of compound XIV and XV were also detected /24/. Ranitidine is a potent inhibitor of gastric secretion after oral administration. It is four to seven times more potent than cimetidine. In doses of 20, 40 and 80 mg, ranitidine reduces hydrogen output by 29%, 50%, and 70%, and gastric secretion volume by 21%, 37%, and 47%. With the same doses it also reduces pepsin activity by 8%, 50% and 49%. Serum concentration of 0.08 μg/ml of ranitidine reduces gastric acid output by 50% /25,26/. Following oral and parenteral administration ranitidine blood concentration curve has a pronounced secondary peak. After oral administration in humans, the first peak in the plasma occurs within 1.1$\pm$0.4 h, and the second peak within 3$\pm$0 h. These peak plasma concentrations are not influenced by food /27/. Following intravenous administration there is a biexponential decline in the plasma levels from 576$\pm$56 ng/ml after 4 min to 10$\pm$2 ng/ml after 8 h. In healthy subjects the cere-

brospinal fluid concentration of ranitidine is one twentieth, to one-thirtieth of that in the plasma sampled at the same time. The distribution half-life of ranitidine is 6.1 ± 0.9 min., elimination half-life being 1.9 ± 0.1 h, the volume of distribution is $96-115 \pm 7$ l, and systemic plasma clearance is 709 ± 62 ml/min. The reported bioavailability after a single dose was between about 40 and 88%, but mostly around 50% /28,29,30/. In the research of the effects on hepatic drug metabolism, it has been found that ranitidine does not inhibit the microsomal drug oxidative function /31/. Ranitidine is 15% protein bound. Half of the oral dose of ranitidine is readily absorbed, and half of the absorbed amount is found unchanged in the urine. Only 1.3 ± 0.3% of the intravenous dose and 2.6 ± 0.2% of the oral dose is converted into the desmethyl metabolite.

8. Identification and Determination in Body Fluids and Tissues

Ranitidine may be determined in the serum, plasma, and urine by high pressure liquid chromatographic analysis /32,33,24/. Martin, et al. /21/ used the on-line high performance liquid chromatography - mass spectrometry for the identification and structure determination of ranitidine and its metabolites in the urine. The separation of ranitidine and its metabolites is usually carried out by extraction of the biological medium with methylene chloride from an aqueous alkaline solution (2M NaOH or 5M KOH), followed by mixing, addition of an internal standard and centrifugation /33/. The addition of 10% isopropanol to methylene chloride increased the recovery of ranitidine in this extraction procedure /32/. The organic layer was then evaporated to dryness under nitrogen at 45°C. When treated in this way, the dry residue was stable for 7 days at -20°C. Before chromatography, the residue was dissolved in a methanol-dibasic ammonium phosphate mixture /33/. Ranitidine was also assayed in the urine by HPLC procedure using direct injection and no internal standard /24/.



XVI

During usual HPLC analyses metiamide /32/ and N-methyl--N'-[3-[(3-dimethylaminomethyl)phenoxy]propyl]-2-nitro--1,1-ethenediamine hydrochloride XVI /33/ were used as internal standards. The columns used for HPLC were the reverse phase μ Bondpak C-18 (Waters Associates), Spherisorb ODS (Phase Separations, Clwyd, Great Britain) and Spherisorb S5 CN. An example of the conditions of analysis is shown in Table VI /32/.

Table VI. Conditions of HPLC analysis

Parameter	Assay conditions required
Mobile phase	92/8 mixture Reagent A/Reagent B
Column	μ Bondpak C-18
Temperature	ambient temperature (2o-25 °C)
Pressure	1ooo - 3ooo psi
Absorbance full scale	o.oo5
Flow rate	2 ml/min
Wavelength	228 nm
Internal standard	metiamide
Chromatography time	8 min

A - ranitidine, B - metiamide

9. Identification and Determination in Pharmaceuticals

For the determination of ranitidine hydrochloride content in a tablet dosage form we recommend the following procedure /13/: Crush 2o tablets in a mortar. Quantitatively transfer the mass of powder equivalent to 1o mg of ranitidine hydrochloride into a 25o cm^3 volumetric flask. Add 1oo cm^3 of water and shake the resulting suspension automatically for 2o minutes. Make up to volume, mix well and centrifuge 2o ml aliquot at 2ooo G for 5 minutes. Pipet 1o cm^3 of the clear supernatant into a 1oo cm^3 volumetric flask and make up to volume. Measure the absorbance of the solution at 313 nm against water and compare it to that of an appropriate standard solution. Calculation:

$$C_{13}H_{23}ClN_4O_3S \text{ contents, mg per tablet} = \frac{A_u \cdot M_s}{A_s \cdot M_u} \cdot ut$$

ut - the average mass of one tablet

For other symbols see chapter 5.

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measurements.

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STRYCHNINE

Farid J. Muhtadi and Mohamed S. Hifnawy

Introduction

Strychnine is an indole alkaloid occurs in numerous *strychnos* plants of the family *Loganiaceae*. The most important of which are the seeds of *strychnos nux-vomica* L. and the beans of *strychnos ignatii Berg.*

These species contain up to 5.3% of the total alkaloids of which approximately one-half is strychnine.

Strychnine was first discovered in 1817 by the French Pharmacists, Pierre Pelletier and Joseph Caventou, and these workers were also responsible for the first isolation of brucine in 1819.

Structural investigations of strychnine were begun by Hanssen and Tafel and continued by Leuchs, Perkin, Robinson, Wieland and Woodward. Finally the structure of strychnine was established by Robinson and co-workers in 1946 and confirmed by X-ray crystallographic analysis and total synthesis by Woodward. The alkaloid has no important therapeutic use as it is highly toxic. However, the crude drug nux-vomica is used as a bitter tonic and stimulant (as it is official in certain pharmacopoeias including the B.P. of 1980).

Strychnine is used as a rodenticide for destroying agricultural rodents and predatory animals. Occasionally, domestic animals and man are poisoned by this agent.

Strychnine is CNS stimulant, it stimulates the spinal cord. It is also a powerful convulsant which produces characteristic convulsions. Sometimes, strychnine is used for the adulteration of street drugs.

1. Description

1.1 Nomenclature

1.1.1 Chemical Name

Strychnidin-10-one

1.1.2 Generic Name

Strychnine

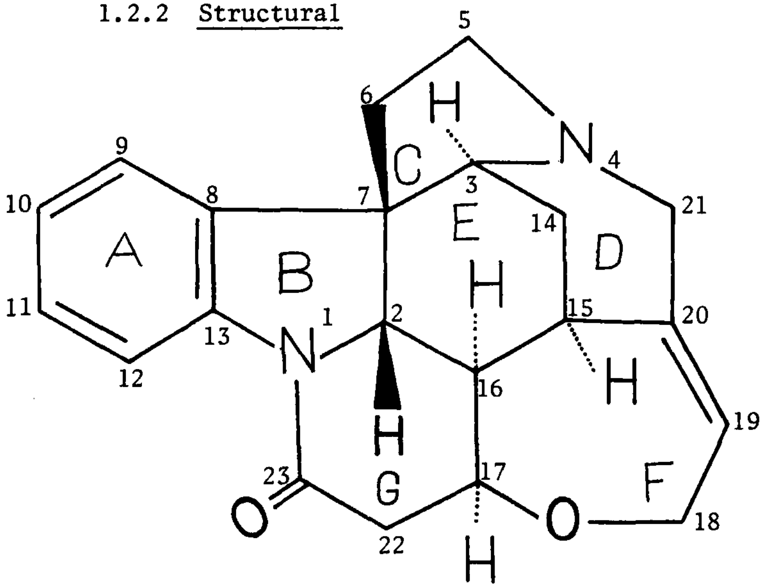
1.2 Formulae

1.2.1 Empirical

$C_{21}H_{22}N_2O_2$ (strychnine)

$C_{42}H_{46}N_4O_8S$ (strychnine sulfate)

1.2.2 Structural



Several structures have been proposed for strychnine, these include, Perkin and Robinson structure of 1910(1), Perkin and Robinson structure of 1929(2), Robinson structure of 1932 (3,4), Leuchs structure of 1932 (5). The currently accepted structure of strychnine was finally established in 1946 by Robinson et al. (6) and was confirmed by the total synthesis of strychnine which was carried out by Woodward et al (7-9). The absolute structure was deduced by extensive X-ray crystallographic studies which were achieved by several authors (10-13).

1.2.3 CAS Registry No.

[57-24-9] strychnine
[60-41-3] strychnine sulfate

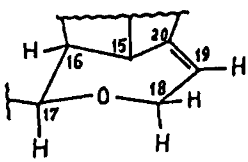
1.2.4 Wiswesser Line Notation

T6 G656 B7 C6 -E5 D
5ABCEF -A&FX MÑQ
-O VN NH-UHJ (14)

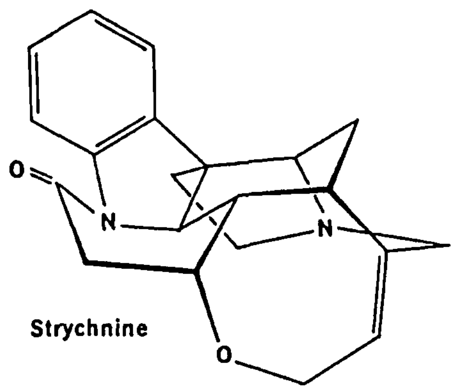
1.2.5 Stereochemistry

The absolute stereochemistry has been deduced

from combinations of massive chemical degradations and correlations (15-18), X-ray crystallographic studies (10-13), ^{1}H-and carbon-13 nuclear magnetic resonance spectral data (19,20). From these studies, it has been established that D and G rings have the boat conformation, while E and F rings have the chair conformation. H atom at C_2 and carbon 7 are above the plane of the molecule (β-atoms), H atoms at C_3, C_{15}, C_{16} and C_{17} lie below the plane of the molecule (α-atoms). Hydrogens at C_3 and that at C_{15} are equatorial, while H at C_2 and that at C_{16} are axial within the chair conformation of ring E.
The conformation of strychnine molecule is presented in I. Recent studies (20) has favored a chair conformation for the seven-membered tetrahydrooxepin ring F (II).



II



I

1.3 Molecular Weight

334.40 (strychnine)
766.92 (strychnine sulfate)

1.4 Elemental Composition

C, 75.42%; H, 6.63%; N, 8.38 %;
O, 9.57%. (strychnine)

C, 65.78%; H, 6.05%; N, 7.31%;
O, 16.69%; S, 4.18%. (strychnine sulfate)

1.5 Appearance, Color, Odor and Taste

Orthorhombic, sphenoidal prisms (from alcohol) (21), or translucent colorless crystals or white crystalline powder (22), odorless and has a very bitter taste. (strychnine)

Colorless crystals or white crystalline powder, odorless and has a bitter taste, effloresces in a dry air (strychnine sulfate).

1.6 Dissociation Constant

pK_1 at 20° : 6.0; pK_2 : 11.7 (21)

1.7 pH Range

pH of a saturated solution of strychnine is 9.5, pH of a strychnine sulfate solution (1:100) is 5.5 (21).

2. Physical Properties

2.1 Melting Range

268-290° Depending on the speed of heating (21)

270-271° Slow heat (23)

strychnine sulfate (anhydrous) : about 200° (21).

2.2 Solubility

One gram dissolves in 6400 ml water, 3100 ml boiling water, 150 ml alcohol, 35 ml boiling alcohol, 5 ml chloroform, 180 ml benzene. (strychnine)

One gram dissolves in 35 ml water, 7 ml boiling water, 81 ml alcohol, 26 ml alcohol at 60°, 220 ml chloroform and in 6 ml glycerol (strychnine sulfate).

2.3 Optical Rotation

$[\alpha]\ D^{18}$ - 139.3° (chloroform) (21)

$[\alpha]\ D^{20}$ - 104° (c = 0.5 absolute alcohol)

$[\alpha]\ D$ - 109.9° (80% ethanol) (23).

(all above data are for strychnine)

The specific rotations for both strychnine in chloroform and strychnine sulfate in water were determined as 20 mg/ml solutions using a Perkin Elmer Polarmatic model 241 MC and found:-

$[\alpha]D^{25}$ - 142.6° (strychnine)

$[\alpha]D^{25}$ - 25.1° (strychnine sulfate)

2.4 Crystal Structure

The crystal structure of strychnine was determined by X-ray diffraction, which was achieved by several authors (10-13).
The crystals of strychnine hydrobromide, $C_{21}H_{22}N_2O_2 \cdot HBr \cdot 2H_2O$ are orthorhombic with space group $P2_12_12_1$ and with cell dimensions a=7.64, b=7.70 and c=33.20A° (10,11). Each bromine atom is bonded to a strychnine molecule through the basic nitrogen atom (N_2).
While the crystals of strychnine sulfate pentahydrate are monoclinic with space group C_2 and cell edges a=35.85±0.05, b=7.56±0.01, c=7.84±0.01A° (12). It has been shown that there are four molecules of strychnine in the unit cell (11,12).
The study revealed that the strychnine molecule has a configuration identical with that deduced by other methods.
Peerdeman (13) by recalculating values of the intensities of reflexions of strychnine hydrobromide dihydride, has deduced the absolute configuration of natural strychnine as presented in Fig. 1.
The crystal structure of strychnine sulfate pentahydrate is presented in Fig. 2 (12). Intramolecular bond lengths of strychnine hydrobromide (11) are tabulated in table 1 and shown on strychnine molecule.

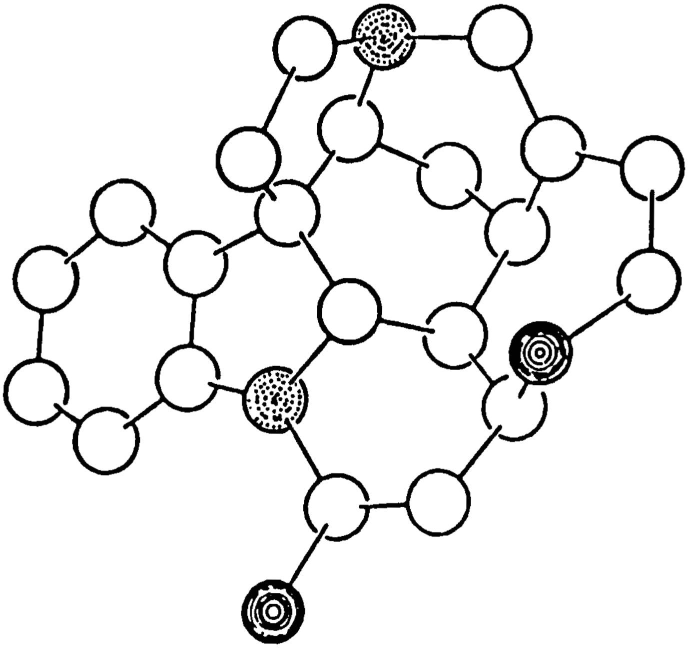


Fig. 1: The Absolute Configuration of Strychnine.

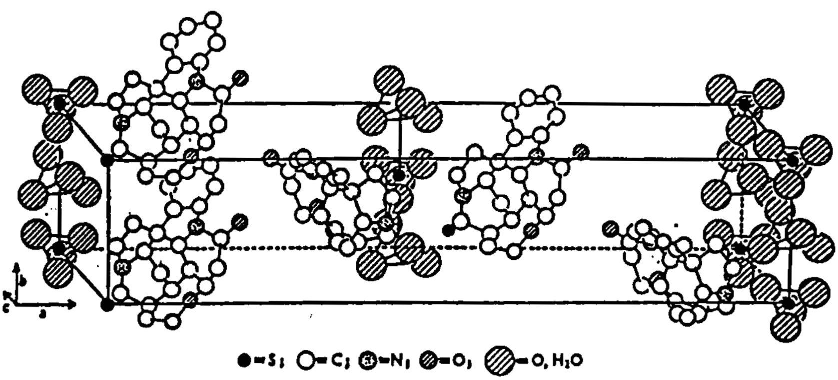
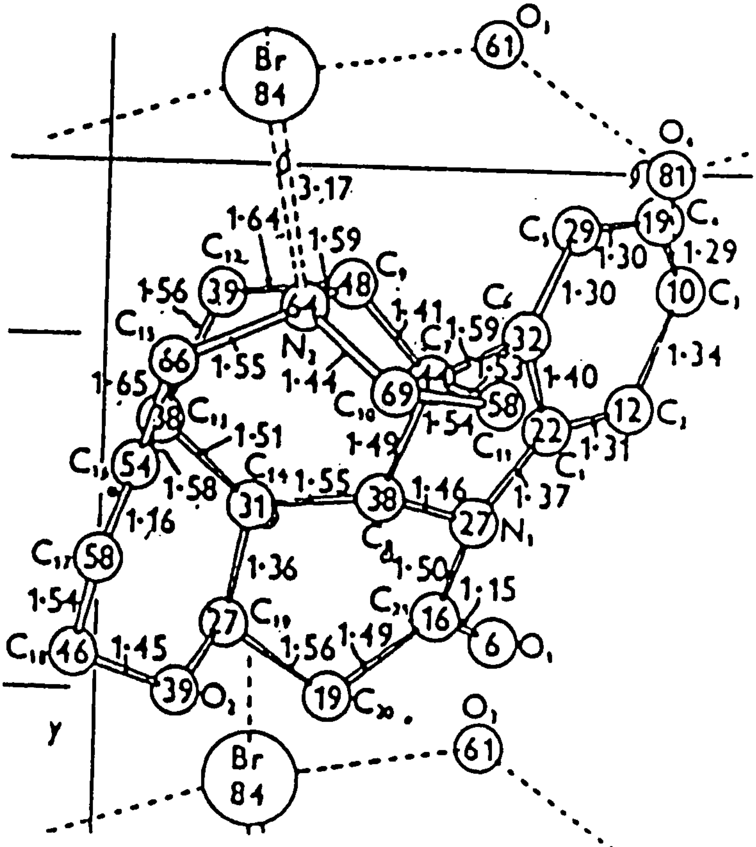


Fig. 2: The Crystal Structure of Strychnine Sulfate.

Table 1. Intramolecular Bond Lengths (Å) of Strychnine Hydrogen Bromide

$C_1 - N_1$	1.37	$C_2 - C_3$	1.34	$C_{12} - C_{13}$	1.56
$C_8 - N_1$	1.46	$C_3 - C_4$	1.29	$C_{13} - C_{14}$	1.51
$C_{21} - N_1$	1.50	$C_4 - C_5$	1.30	$C_{13} - C_{16}$	1.58
$C_9 - N_2$	1.59	$C_5 - C_6$	1.30	$C_{14} - C_{19}$	1.36
$C_{10} - N_2$	1.44	$C_6 - C_7$	1.59	$C_{15} - C_{16}$	1.65
$C_{15} - N_2$	1.55	$C_7 - C_8$	1.49	$C_{16} - C_{17}$	1.16
$C_{21} - O_1$	1.15	$C_7 - C_9$	1.41	$C_{17} - C_{18}$	1.54
$C_{18} - O_2$	1.45	$C_7 - C_{11}$	1.53	$C_{19} - C_{20}$	1.56
$C_{19} - O_2$	-	$C_8 - C_{14}$	1.55	$C_{20} - C_{21}$	1.49
$C_1 - C_2$	1.31	$C_9 - C_{12}$	1.64	N_2 - Br	3.17
$C_1 - C_6$	1.40	$C_{10} - C_{11}$	1.54		



2.5 Spectral Properties

2.5.1 Ultraviolet Spectrum (UV)

The UV spectrum of strychnine in methanol (Fig. 3) was scanned from 190 to 400 nm using DMS Varian Spectrometer. It exhibited the following UV data (Table 2).

Table 2. UV Characteristics of Strychnine

λmax. nm	log ε	A (1%, 1 cm)
205	-	-
254	4.10	377
280	3.64	131.4
290	3.54	104

Other reported UV spectral data for strychnine in ethanol λmax. 255 nm (E 1%, 1 cm = 377) (24); in sulfuric acid λmax. at 255 nm (E 1%, 1 cm = 315) (24); λmax. for strychnine in ethanol, 254, 278, 288 mμ (log ε 4.10, 3.63, 3.53 (21).

2.5.2 Infrared Spectrum (IR)

The IR spectrum of strychnine as KBr disc was recorded on a Perkin Elmer 580 B Infrared Spectrometer to which an infrared data station is attached (Fig. 4).
The structural assignments have been correlated with various frequencies (Table 3).

Table 3. IR Characteristics of Strychnine

Frequency cm^{-1}	Assignment
2950-2800	CH stretch
1672	C=O substituted amide
1600, 1485	C=C aromatic
1150, 1110, 1055	ether linkage ∖O∕
770	4-adjacent aromatic hydrogens

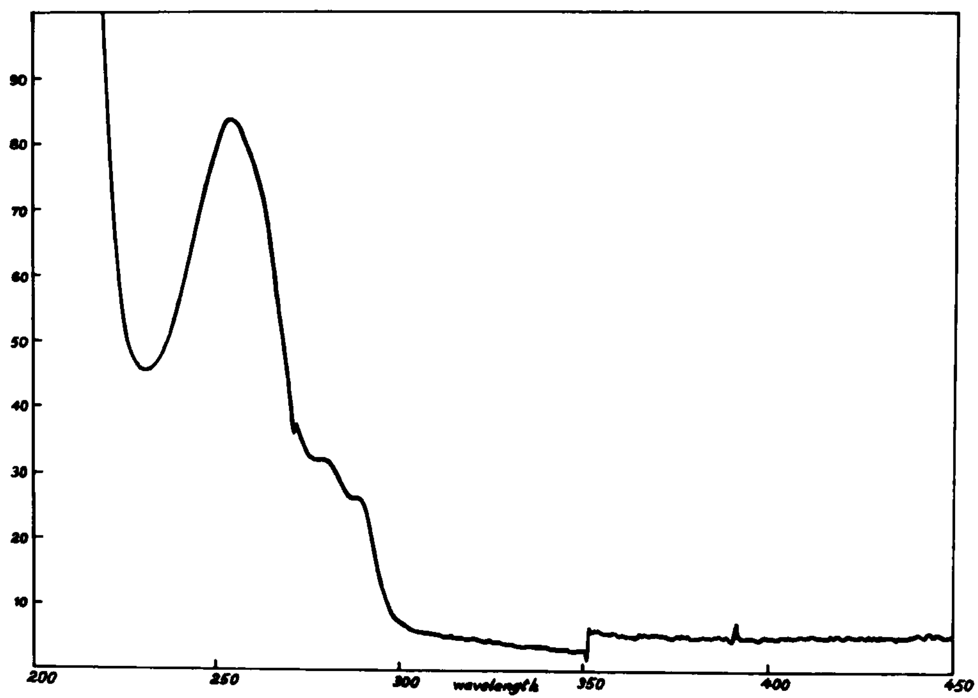


Fig.3: The UV spectrum of strychnine in methanol

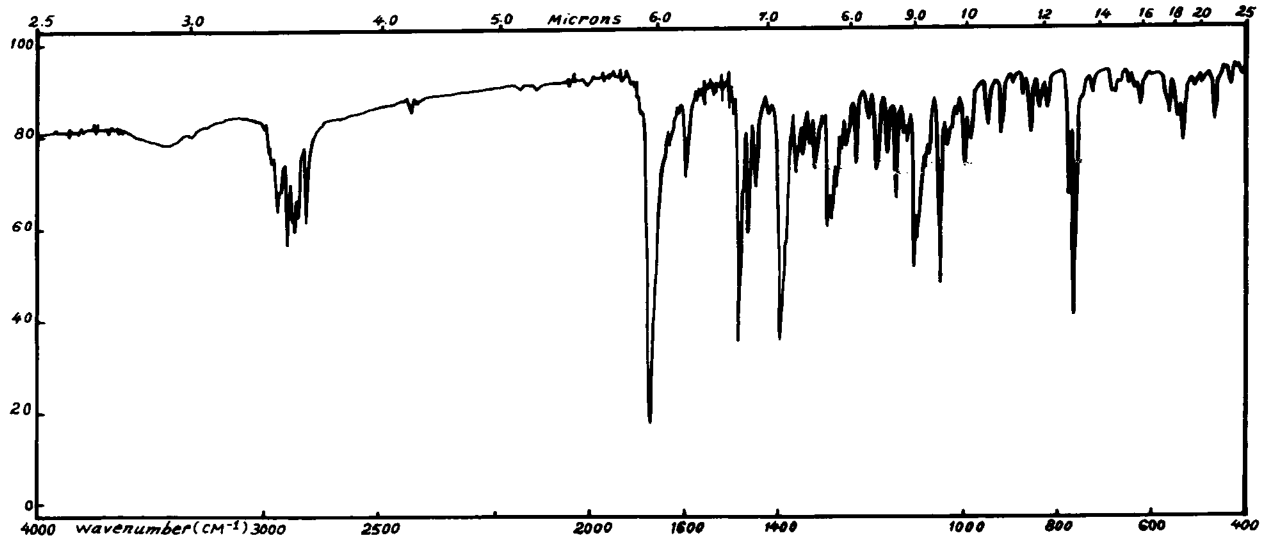


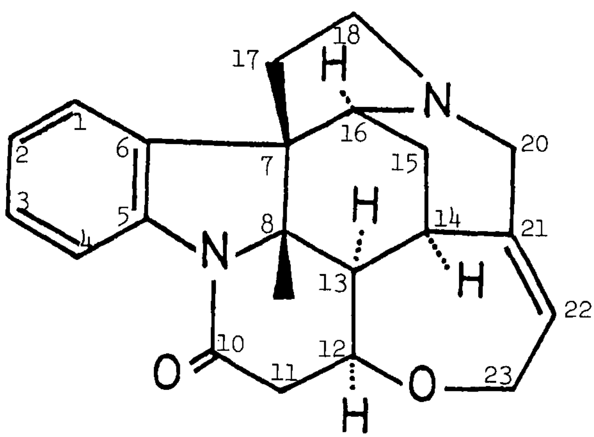
FIG. 4. THE IR SPECTRUM OF STRYCHNINE KBR. DISC

The IR exhibited the following other characteristic bands :- 1465, 1450, 1395, 1365, 1350, 1295, 1280, 1240, 1190, 1170, 1000, 990, 950, 925, 860, 780, 540, 470 cm^{-1}. Other IR data for strychnine have been reported (9,14,24).

2.5.3 Nuclear Magnetic Resonance Spectra

2.5.3.1 Proton Spectra (PMR)

The PMR spectra of strychnine in $CDCl_3$ and in TFA (trifluoroacetic acid) were recorded on a Varian T-60A, 60 MHz NMR Spectrometer using TMS (Tetramethylsilane) as an internal reference. The spectra are shown in Fig. 5 and 6 respectively. The following structural assignments have been made (Table 4).



The PMR of strychnine in $CDCl_3$ using 250 MHz spectrometer has been published earlier (19). The spectrum obtained by this instrument (Fig. 7) afforded better resolution for structural assignment particularly in the region 1.2 - 4.3 ppm.

The reported PMR data of strychnine (19) are also presented in table 4 along with our found PMR characteristics.

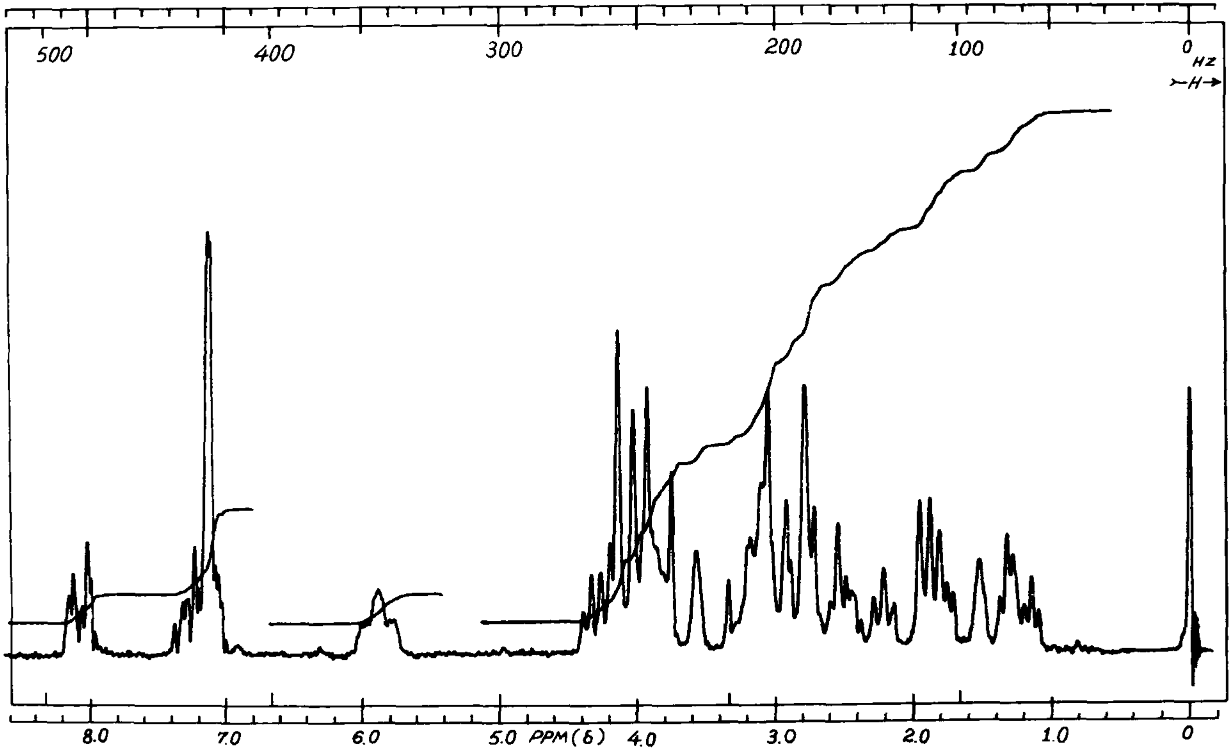


FIG. 5. THE PMR SPECTRUM OF STRYCHNINE IN $CDCl_3$.

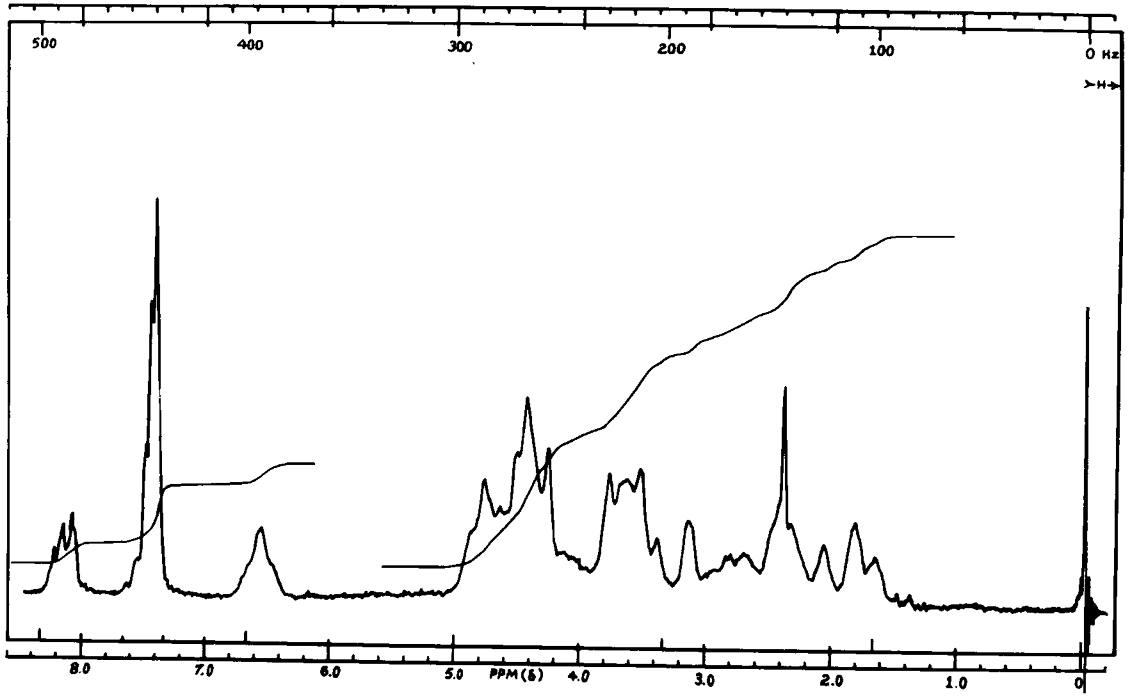


Fig. 6: The PMR spectrum of strychnine in TFA.

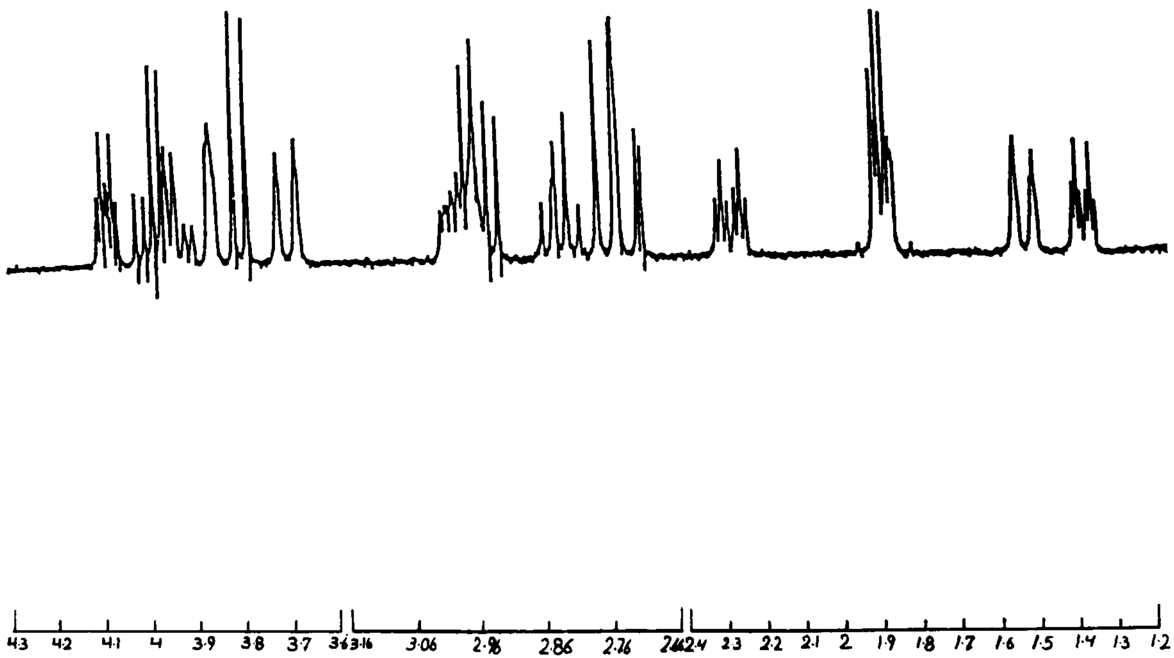


Fig.7: The PMR of strychnine in $CDCl_3$ using
250 MHz spectrometer.

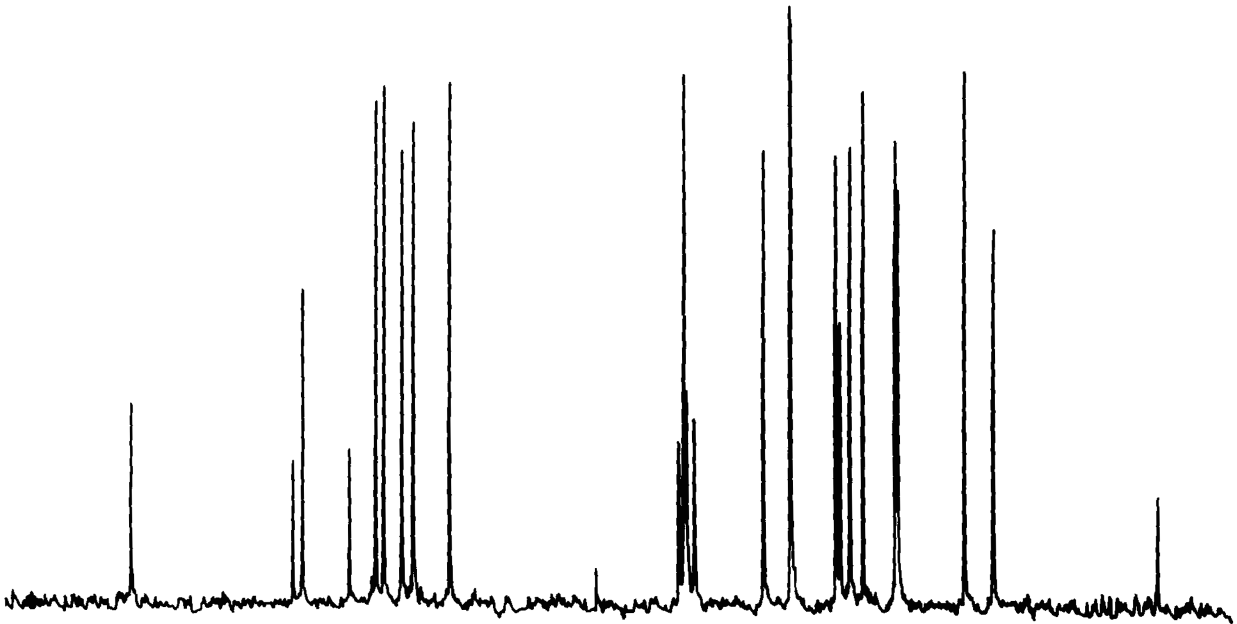


Fig.8: ^{13}C-NMR Noise-Decoupled Spectrum of strychnine.

Table 4. PMR Characteristics of Strychnine

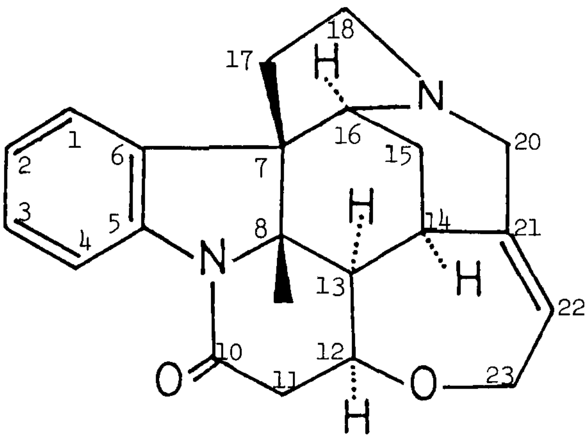
Proton at	Chemical Shift (ppm)		
	$CDCl_3$ Reported	$CDCl_3$ Found	TFA
C_1,C_2,C_3 (aromatic)	7.14,7.07,7.23	7.20(m)	7.40
C_4 (aromatic)	8.08	8.07(2q)	8.13
C_8	3.84	3.88(s)	
C_{11}	3.10 2.65	3.08(s) 2.60(s)	4.23
C_{12}	4.26	4.27(q)	4.76
C_{13}	1.25	1.25(t)	1.74
C_{14}	3.12	-	
C_{15}	1.43 2.33	1.52(d) 2.35(t)	2.03 2.76
C_{16}	3.92	3.88(s)	
C_{17}	1.86, 1.87	1.85(m)	2.38
C_{18}	2.86 3.18	2.83(q) 3.16(s)	3.66
C_{20}	2.71, 3.69	3.6 (s)	
C_{22} (vinylic)	5.88	5.87(bt)	6.53
C_{23}	4.04 4.12	4.04(s) 4.13(s)	4.40

s = singlet, d = doublet, t = triplet, m = multiplet, q = quartet, 2q = 2 quartets, bt = broad triplet.

Some stereochemical correlations in strychnine molecule were deduced from the coupling constants of its protons (19,20).

2.5.3.2 ^{13}C-NMR

The ^{13}C-NMR noise-decoupled and off-resonance spectra are presented in Fig. 8 and Fig.9 respectively. Both were recorded over 5000 Hz in $CDCl_3$ on a Joel FX-100 NMR Spectrometer, using a 10 mm sample tube and tetramethylsilane (TMS) as an internal reference standard at 20°. The carbon chemical shifts are assigned on the basis of additivity principals and the off-resonance splitting pattern (Table 5).
Assignments of all 21 carbons of strychnine are consistent with those



of Wenkert et al. (20) and Verpoovte et al (26).

Table 5. Carbon Chemical Shifts of Strychnine

Carbon No	Chemical Shift [ppm]	Carbon No	Chemical Shift [ppm]
C_1	122.17 (d)	C_{13}	48.14 (d)
C_2	124.05 (d)	C_{14}	31.52 (d)
C_3	128.39 (d)	C_{15}	26.77 (t)
C_4	116.06 (d)	C_{16}	60.00 (d)

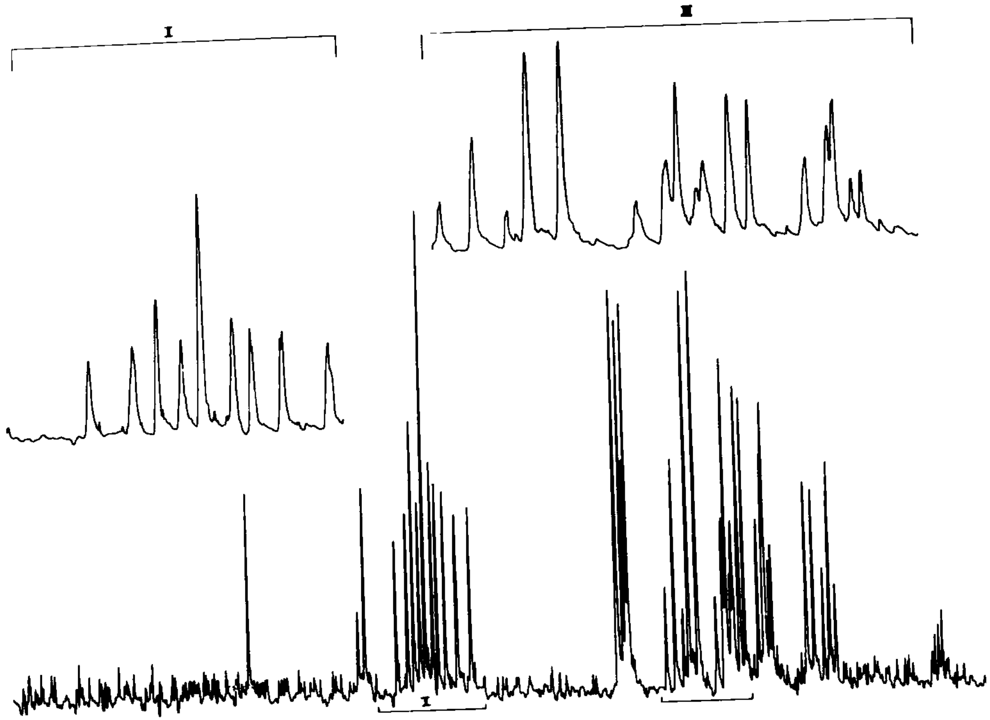


Fig.9: ^{13}C-NMR Off-Resonance Spectrum of strychnine.

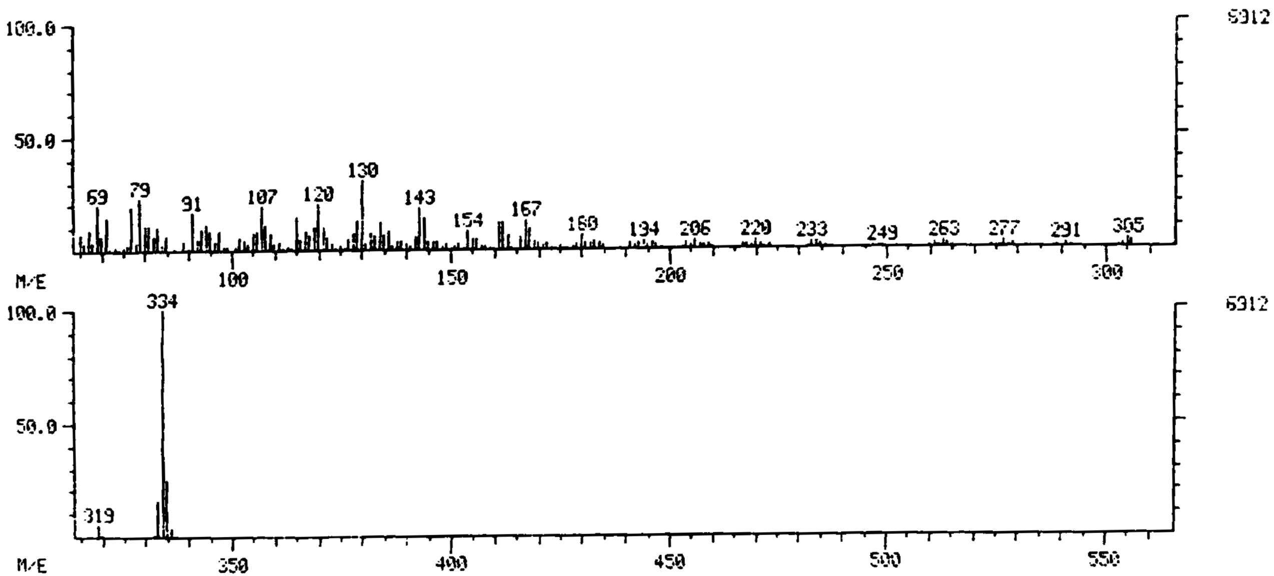


FIG. 10 : THE MASS SPECTRUM OF STRYCHNINE.

Carbon No	Chemical Shift [ppm]	Carbon No	Chemical Shift [ppm]
C_5	142.08 (s)	C_{17}	42.80 (t)
C_6	132.68 (s)	C_{18}	50.25 (t)
C_7	51.84 (s)	C_{20}	52.40 (t)
C_8	60.11 (d)	C_{21}	140.49 (s)
C_{10}	169.14 (s)	C_{22}	127.04 (d)
C_{11}	42.38 (t)	C_{23}	64.52 (t)
C_{12}	77.49 (d)		

Multiplicity symbols, s = singlet, d = doublet, t = triplet.
Other ^{13}C-NMR spectral data of strychnine have been also reported (20,25-27).

2.5.4 Mass Spectrum

The mass spectrum of strychnine is presented in Fig. 10. This was obtained by electron impact ionization (EI) on a Finnigan - Mat 1020 by direct inlet probe at 270°c. The electron energy was 70 eV. The spectrum scanned to mass 550 amu. The spectrum (Fig. 10) shows a molecular ion peak M^+ at m/e 334 with relative intensity of 100% which corresponds to the base peak.
The most prominent fragments and their relative intensities are presented in table 6.

Table 6. Mass Fragments of Strychnine

m/e	Relative Inten. %	m/e	Relative Inten. %
335	24.65 (M^{+1})	130	30.50
334	100.0 (M^{+})	129	12.79
333	16.20 (M^{-1})	120	20.40
167	13.11	115	14.74
162	12.41	108	11.13
144	13.90	107	19.42
143	18.17	94	11.00
134	11.76		

m/e	Relative Inten. %
91	17.07
81	11.18
80	11.10
79	23.50
77	19.13
71	14.55
69	19.99

Other mass spectrum data of strychnine have been also reported (28).

3. Isolation of Strychnine

Strychnine occurs along with brucine in several species of *Strychnos* particularly *S. nux-vomica* and *S. ignatii (Family Loganiaceae)* which contain varying quantities of both alkaloids (0.5 to 5.3%).

Finely powdered nux-vomica is thoroughly moistened with lime water and extracted with hot chloroform till exhausion.

The alkaloids are removed from the solvent by shaking with successive portions of diluted sulfuric acid. The combined acid solution is concentrated. Strychnine can be isolated from brucine by one of the following steps:-

1- The less soluble brucine bisufate crystallizes from the acid concentrated solution first and removed by filteration. Upon neutralization of the mother liquor, strychnine sulfate crystallizes out, and purified. The alkaloid is made from it by precipitation with ammonia solution (29).

2- The concentrated acid solution is rendered alkaline with excess ammonia solution, where strychnine and brucine precipitated together. The precipitate is extracted with 25% ethanol which dissolves the brucine, and leaves the strychnine as insoluble residue. The undissolved strychnine is filtered off (30).

- Strychnine is purified by repeated crystallization from ethanol.

4. Total Synthesis of Strychnine

The total synthesis of strychnine was achieved in 1954 by Woodward and his associates (7, 8). Before this several attempts have been made, but these were unsuccessful. Woodward's synthesis of strychnine stands out as a major synthetic achievement (17,31).

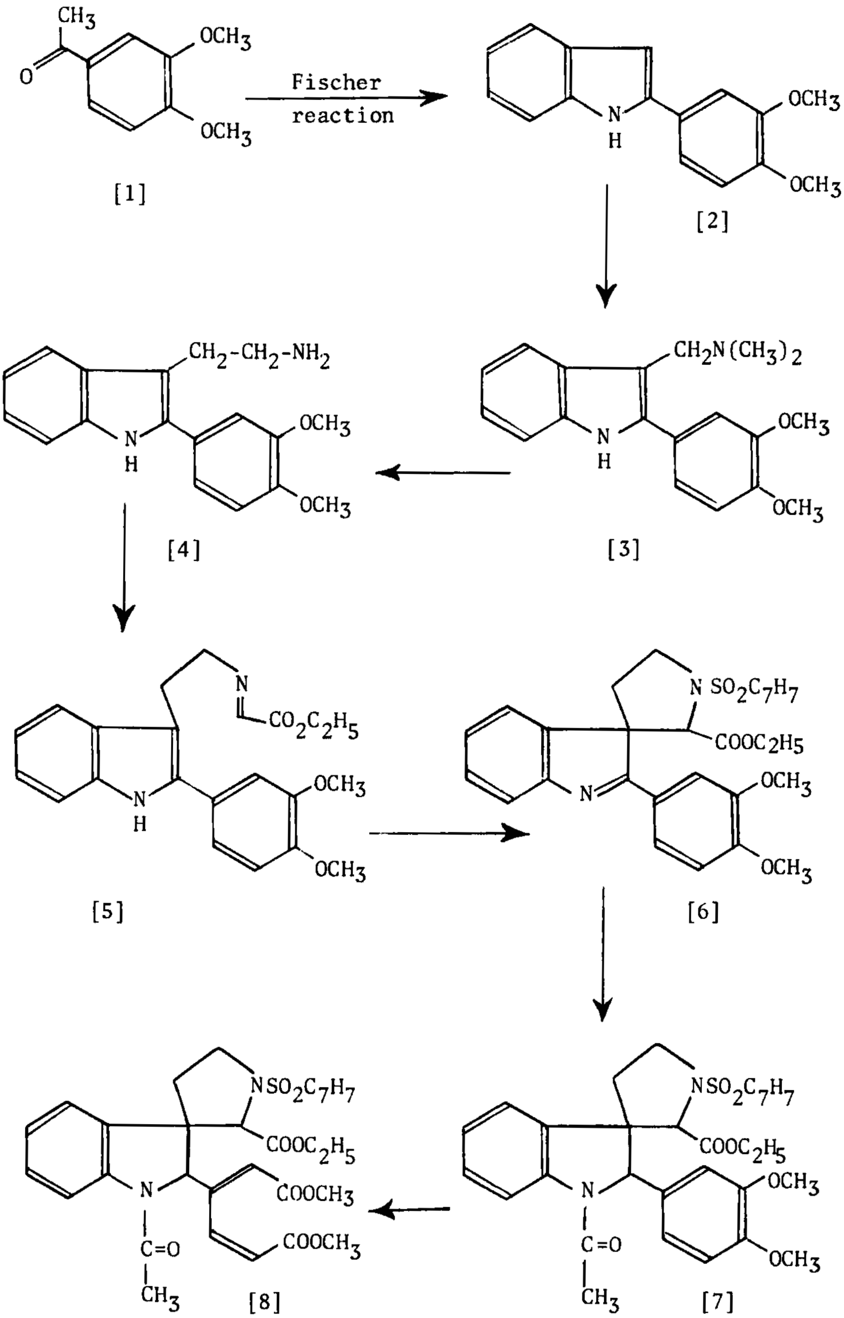
Woodward's total synthesis of strychnine (7-9):-

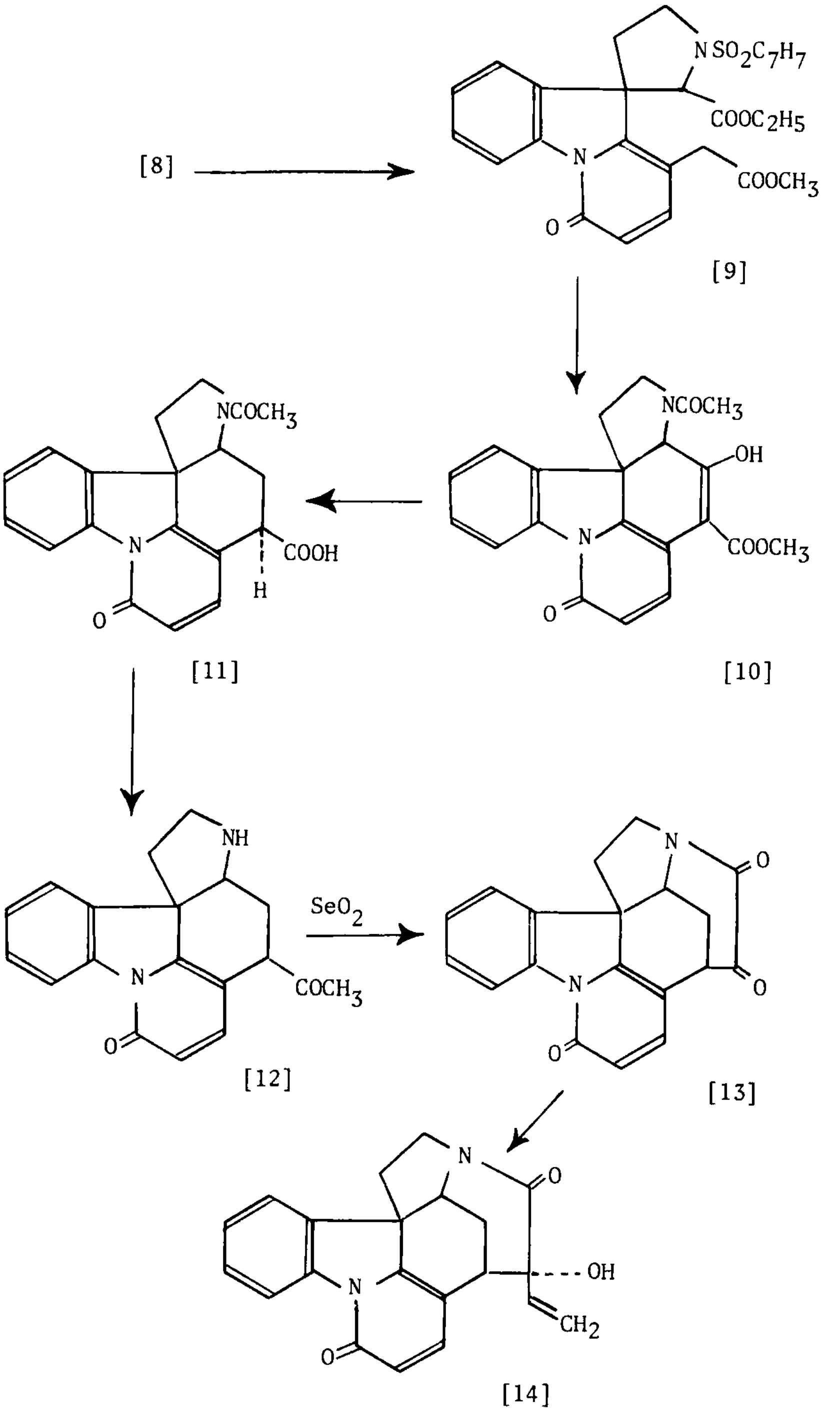
Acetoveratrone [1] and phenylhydrazine are added to polyphosphoric acid and the mixture is warmed to give 2-veratrylindole [2].

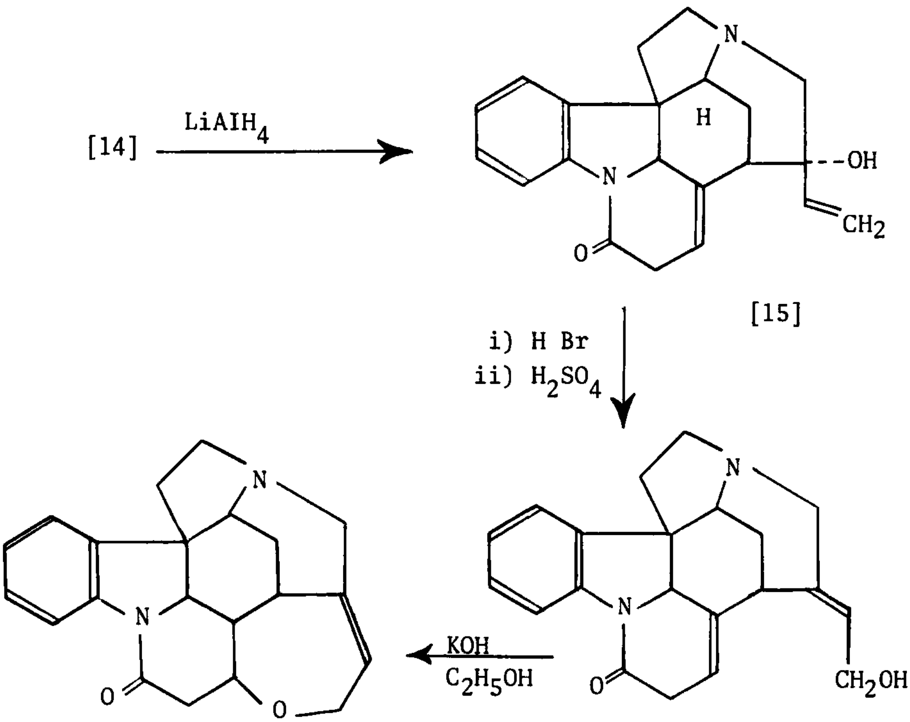
2-veratrylindole [2] is condensed with formaldehyde and aqueous dimethylamine in dioxan and acetic acid to give 2-veratrylgramine [3]. The methiodide of [3] is converted by sodium cyanide in dimethylformamide to the nitrile, which in turn is reduced by lithium aluminium hydride in hot tetrahydrofuran to 2-veratryltryptamine [4]. This is condensed with ethyl glyoxylate in warm benzene to give

the corresponding Schiff's base [5]. The latter is treated with p-toluenesulfonyl chloride in pyridine to produce the indolenine [6]. The indolenine [6] is reduced by sodium borohydride in ethanol to the corresponding indoline which is converted into the N-acetyl-indoline derivative [7] by the action of acetic anhydride and pyridine. [7] is treated with ozone in aqueous acetic acid (ozonolysis) to yield the triester [8]. The triester [8] is reacted with boiling methanolic hydrogen chloride, cleavage occurs to give the pyridone ester [9]. This is treated with hot hydriodic acid in the presence of red phosphorus, followed by esterification, N-acetylation and treatment with sodium methoxide in hot methanol to yield the cyclized product [10]. The latter is treated with p-toluene sulfonylchloride in pyridine to yield O-tosyl derivative which is reacted with sodium benzylmercaptide to give the β-benzylmercaptoester. This upon treatment with deactivated Raney nickel in hot ethanol followed by reduction with hydrogen in the presence of palladium on charcoal gives the cis saturated ester, alkaline hydrolysis is effected followed by acidification to give the trans acid [11]. The acid [11] is resolved with quinidine and refluxed with acetic anhydride and pyridine then hydrolysed with aqueous hydrochloric and acetic acids to produce the aminoketone [12]. Oxidation of [12] with selenium dioxide in ethanol yields dehydrostrychninone [13]. The latter is reacted with sodium acetylide in tetrahydrofuran followed by reduction with hydrogen in the presence of Lindlar palladium (32) to give the corresponding vinyl compound [14]. This is transformed by lithium aluminum hydride in ether into the base [15]. The base [15] is subjected to boiling at 120° with hydrogen bromide in acetic acid in the presence of red phosphorus followed by hydrolysis with boiling aqueous sulfuric acid to yield isostrychnine [16]. This upon treatment with ethanolic potassium hydroxide is converted into strychnine [17].

The Total Synthesis of Strychnine




[8]
$NSO_2C_7H_7$
$COOC_2H_5$
$COOCH_3$
N
O
[9]
$NCOCH_3$
OH
$COOCH_3$
N
O
[10]
$NCOCH_3$
COOH
H
N
O
[11]
NH
$COCH_3$
N
O
[12]
SeO_2
N
O
O
N
O
[13]
N
O
OH
CH_2
N
O
[14]

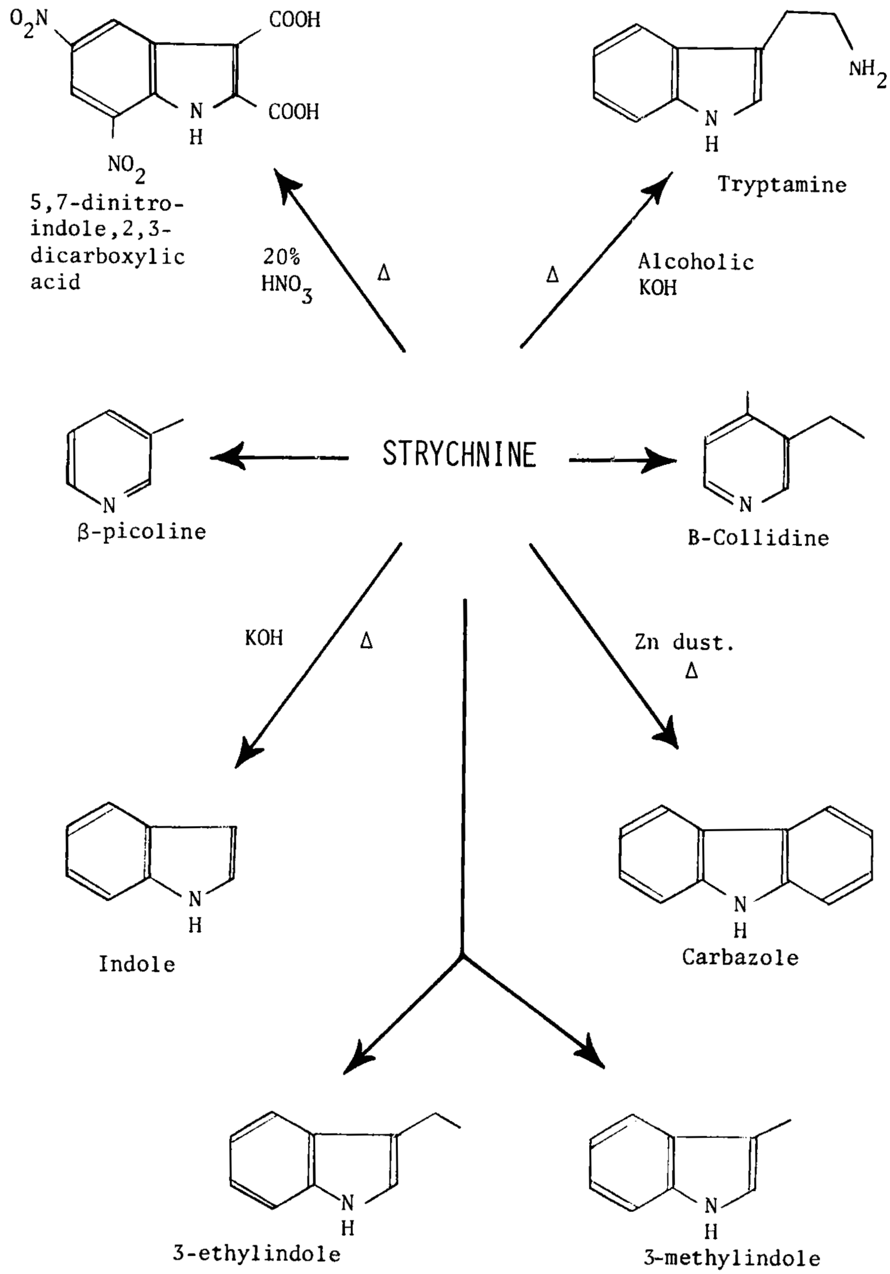


[17] [16]

Degradation

Strychnine molecule can be degraded into several products. The most interesting degradative products are those obtained by alkaline degradation and by the action of 20% nitric acid. Alkaline degradation generate seven simple isolable products of known structures (15,16,17, 31,33,34), while 20% nitric acid treatment affords 5,7-dinitroindole-2,3-dicarboxylic acid (35).
The degradative products are shown below.

Products of the Alkaline Degradation of Strychnine and the effect of 20% nitric acid.



5. Biosynthesis of Strychnine

It has long been assumed that the indolic moiety of the indole alkaloids is derived from the aminoacid "tryptophan" (36-38). Thomas (39) and Wenkert (40) have independently predicted that the non-tryptophan portion of these alkaloids is formed from two mevalonate units to afford a cyclopentane monoterpene. Thomas (41) has further suggested that the glucoside "loganin" could be a key intermediate in the biosynthetic pathway to indole alkaloids. This suggestion has been established upon experimental evidence by several authors (42-50).
It is now known that loganin arises in the plants from mevalonate which is transformed by a series of steps to isopentenyl diphosphate (51) and dimethylallyl diphosphate (52). Combination of these leads to geraniol (53,54), then to loganin and finally to secologanin which condenses with tryptophan to afford intermediate condensate to many indole alkaloids (55).

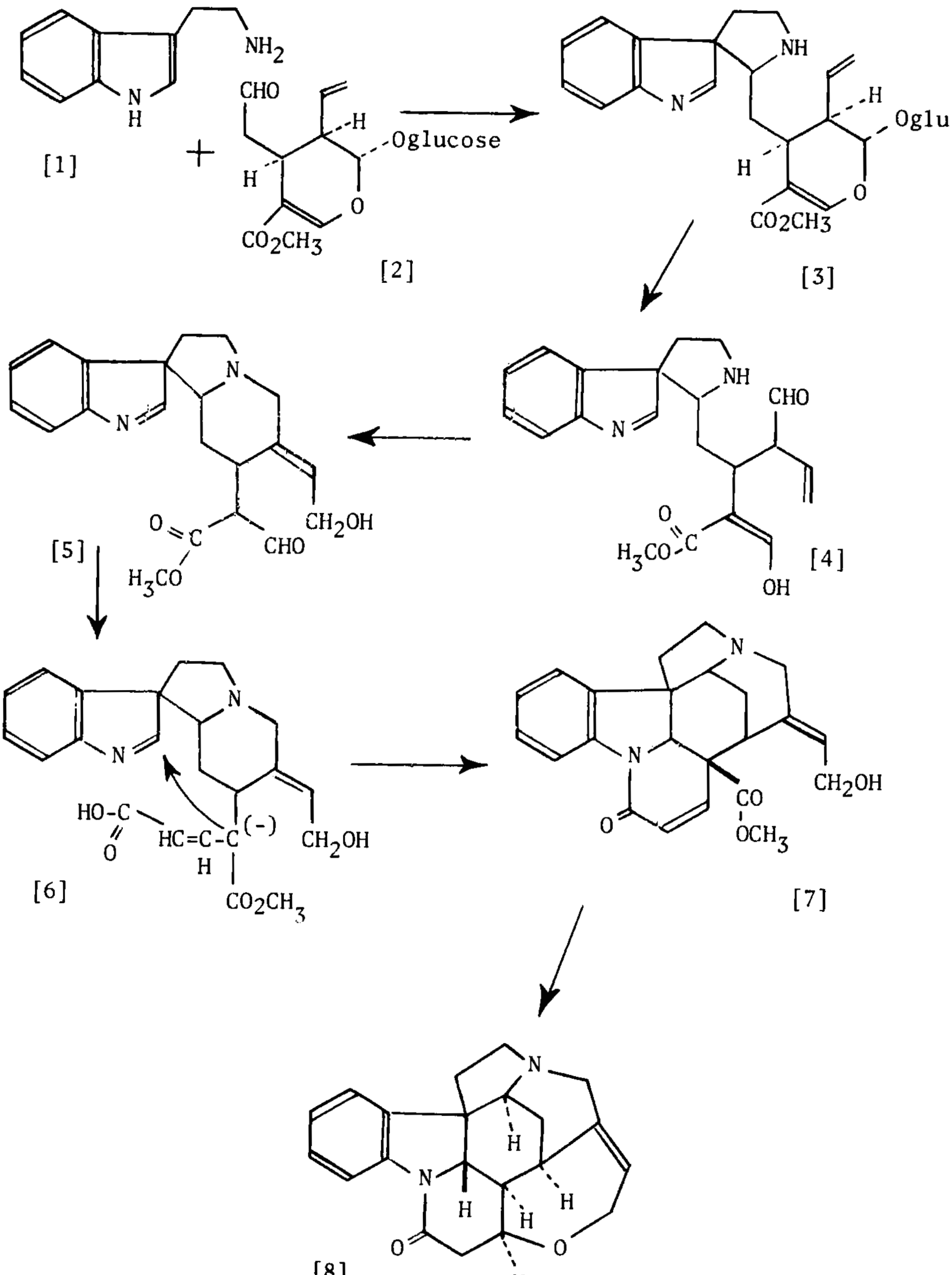
Many radioactive precursors were fed into the plants of *Strychnos nux-vomica*. Several of these were incorporated into strychnine. These include [2-^{14}C]-tryptophan (47), [1-^{15}N]-tryptophan (56,57), [1-^{14}C]-acetate, [2-^{14}C]-acetate (47), [2-^{14}C]-glycine (57-59), [2-^{14}C]-mevalonate (47), [2-^{14}C]-geraniol (47), and [2-^{14}C]-geranylpyrophosphate (47). While other precursors were not incorporated into strychnine and among these are [ar-^{3}H]. Weiland-Gumlich aldehyde and [2-^{14}C]-1-acetyl Weiland-Gumlich aldehyde (47).

The biosynthetic pathway leading to strychnine can be visualized from two different systems (55). The first system involves condensation of tryptamine and secologanin to form a Schiff base which could be attacked intramolecularly by the β-position of the indole nucleus to afford the corresponding spiroindolenins (55). This system is presented in Scheme I.
The other system involves condensation of tryptophan and secologanin at the α-position of the indole neucleus to afford β-carboline system which leads to strictosidine (55). This system is presented in Scheme II (55,60).

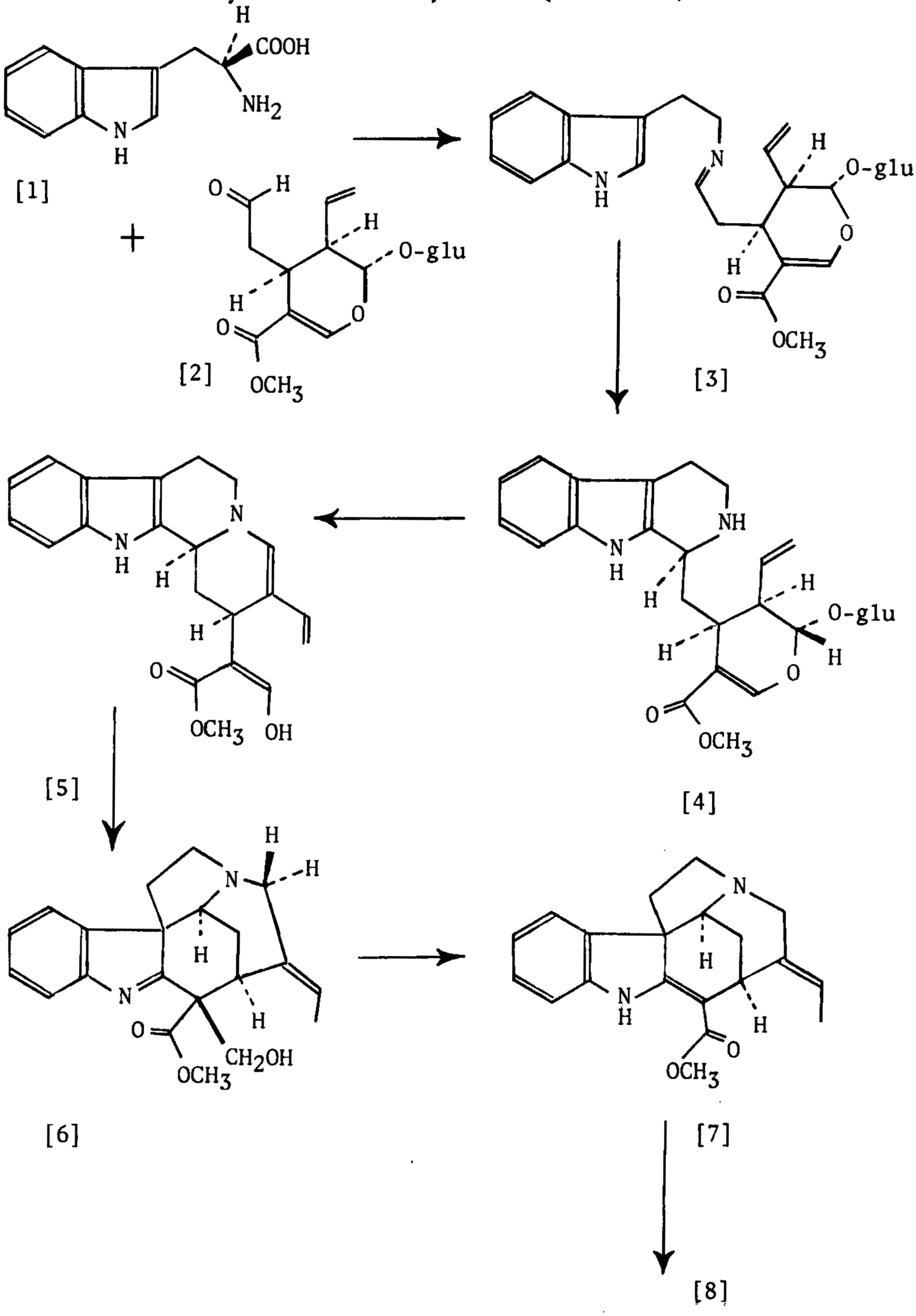
Scheme I

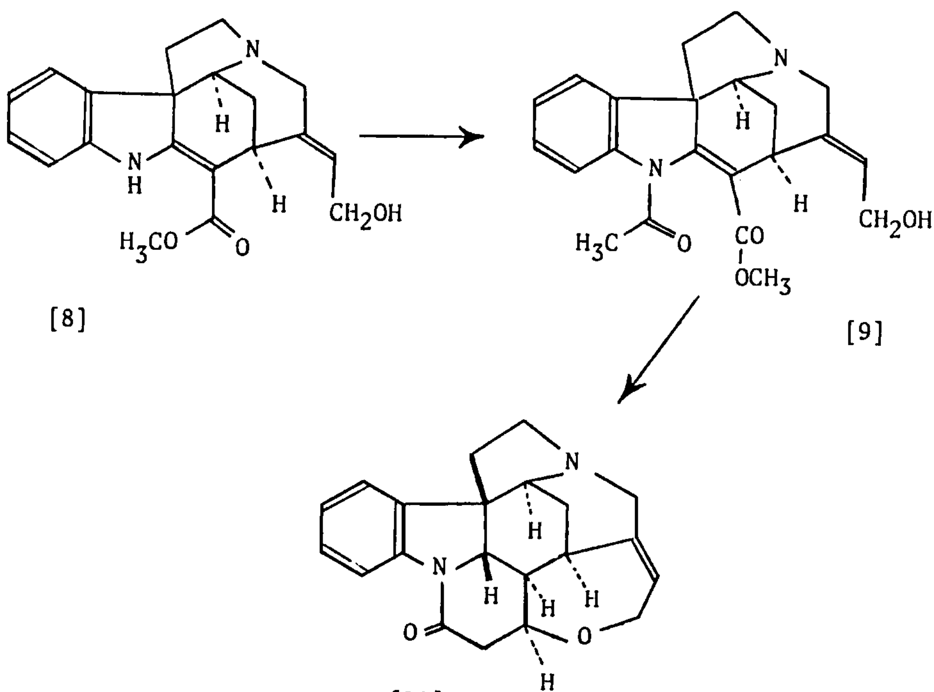
Tryptomine [1] is condensed with secologanin (Mannich condensation) [2] to give the spiroindolenine [3]. This undergoes ring opening to give the intermediate [4]. The latter is transformed into the intermediate [5]. Acetate unit is incorporated into [5] to afford [6]. Intramolecular cyclization gives [7], which upon further cyclization and decarboxylation yields strychnine [8].

Biosynthesis of Strychnine (Scheme I).



Biosynthesis of Strychnine (Scheme II)





[8] [9] [10]

Scheme II

Tryptophan [1] is condensed with secologanin [2] to give the condensate [3]. Cyclization of [3] yields isovincoside (also known as strictosidine) [4]. Further cyclization and ring opening of [4] affords pregeissoschizine [5]. This is transformed into preakuammicine [6] which is converted by a retro-aldol process to akuammicine [7]. Oxidation at the allylic carbon of [7] affords [8]. Incorporation of acetate unit to [8] yields [9] which undergoes reductive cyclization to produce strychnine [10].

6. Metabolism of Strychnine

Strychnine is very rapidly absorbed from the gastrointestinal tract, through mucous membranes, intact skin and from an injection site (22,24,61).
Strychnine travels in both plasma and erythrocytes and approximately 50% of the agent will diffuse into tissues in five minutes (62).
The CNS does not contain higher concentrations than do other tissues (61).
Strychnine is readily metabolised in the liver by microsomal enzymes (61,63), where up to 80% of the dose is oxidized and approximately 10 to 20% of the dose will appear in the 24 hour urine unchanged (24,62).
Strychnine is cumulative and very stable and may be found in cadavers exhumed many years after death (24).

Toxicity

Poisoning by strychnine still occurs particularly from rodenticides or from the adulteration of street-drugs with strychnine. Many cases of accidental poisoning are in children who may swallow or suck strychnine baits. The fatal adult oral dose is about 50 to 100 mg of strychnine, but 30 mg has been found lethal (61).
The main effect of strychnine is on the spinal cord where it inhibits post-synaptic inhibitory control, leading to tetanic convulsions. Death occurs from central respiratory failure.

7. Methods of Analysis

7.1 Identification tests

The following color tests are used to identify strychnine.

- If a trace (few crystals) of strychnine is dissolved in few drops of concentrated sulfuric acid and stirred with a crystal of potassium dichromate, a deep bluish violet color develops, which gradually changes to red and finally to orange yellow color.
- When a trace of strychnine is treated with Mandalin's reagent (Ammonium vanadate in sulfuric acid), a blue color is formed which changes first to purple then to red color (sensitivity 0.05 μg).
- Few drops of fuming nitric acid are added to a trace of strychnine, a yellow color is produced. Brucine gives with nitric acid an intense orange red color.
- Strychnine gives with Vitali's test a brown color (sensitivity 0.5 μg). The test can be performed as follows:
  Few crystals of strychnine are dissolved in few drops of nitric acid and the yellow solution is evaporated to dryness on a water bath, when alcoholic potash is added to the residue, a brown to purple color is developed.
- When O_ Tosyl-p-phenolsulfonic acid is added to certain alkaloids including strychnine, a precipitate is obtained which is crystallized from water to give crystals with m.p. of 242-244° (64).
- When 3-nitro-4-chloro-5-methylbenzene sulfonic acid is added to some alkaloids including strychnine, a precipitate of the salt is formed, upon crystallization from water, the crystals melt at 265-266° (64).
- A simple, rapid and sensitive technique for the detection of strychnine among other alkaloids and psychotropic amines in physiological fluids such as saliva and urine was described, pot. acid phthalate buffer 0.1 M, pH 4.5, $CHCl_3$, and NaOH 0.05 N were used for extraction and bromophenol blue 0.04% in MeOH for color production. The blue color in alkaline medium was very visible. The method was recommended for toxicology (65).

7.2 Microcrystal Formation

Strychnine hydrochloride was dissolved in water (10 mg in 10 ml), 1 to 2 drops of this solution was treated with the reagent on a microscopical glass slide. After specific time, the crystals were microscopically examined. The crystal formation is presented in table 7.

Table 7. Microcrystal Formation of Strychnine

Plate	Reagent	Time	Shape of the Crystal
1	Mayer's	2-3 min.	minute plates in star like arrangement
2	Platenic chloride	2-3 min.	broad plates, rosette arrangement
3	Mercuric chloride	10 min.	fine curved hairy with specific arrangement
4	Wagner's	15 min.	fine short rods
5	Dragendorff	2-3 min.	rods in cluster form
6	Marm's	immediate	rosetts of plates, enlarged by time
7	Sodium carbonate	10 min.	long rods in rosette shape and free arrangement
8	Sodium phosphate	10-15 min.	cluster of large plates
9	Potassium chromate	immediate	thin radiating rods
10	Potassium iodide	3-5 min.	broad plates, radiating form

7.3 Titrimetric Methods

The official methods of determination of strychnine in formulations or in powdered nux vomica and its preparations, by titrimetric methods are described in the Egyptian Pharmacopoea (E.P.) 1963 and 1972 (66,67).

7.3.1 Aqueous Titration

The E.P. 1963 method for determination of strychnine in nux vomica galenicals is based on the oxidative destruction of brucine with HNO_3 and the acid base titration of strychnine. Transfer accurate volume (20 ml) of nux vomica extract to a separator, dilute with about 20 ml H_2O, acidify with dilute H_2SO_4 (about 5 ml) and extract with $CHCl_3$ (2x20 ml). Receive the

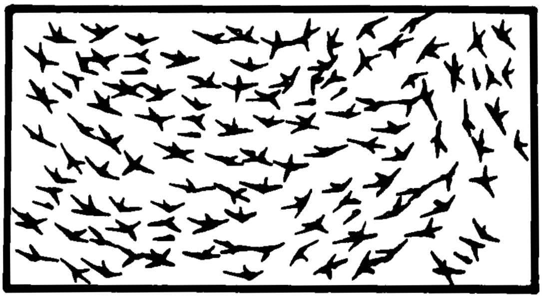


Plate 1 : Microcrystals of strychnine with Mayer's reagent

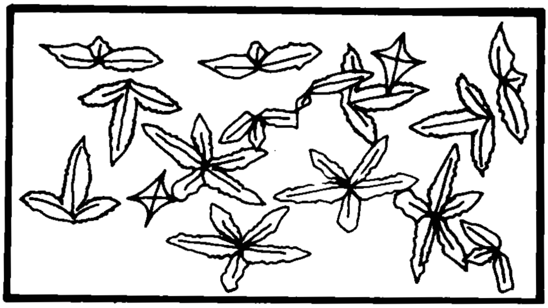


Plate 2 : Microcrystals of strychnine with Platinic chloride

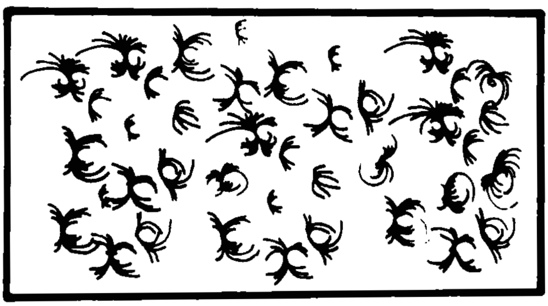


Plate 3 : Microcrystals of strychnine with Mercuric chloride

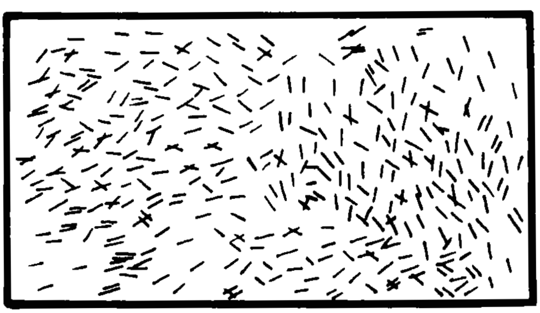


Plate 4 : Microcrystals of strychnine with Wagner's reagent

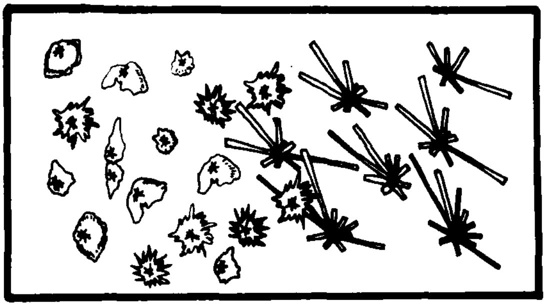


Plate 5 : Microcrystals of strychnine with Dragendorff's reagent

Plate 7 : Microcrystals of strychnine with Sodium carbonate

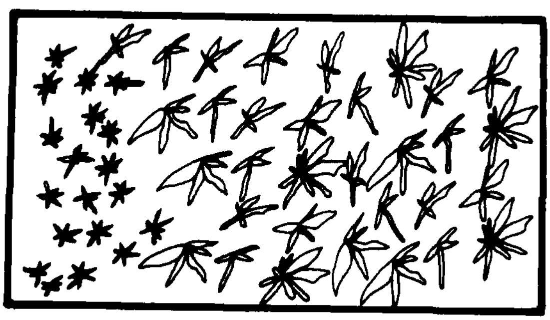


Plate 6 : Microcrystals of strychnine with Marm's reagent

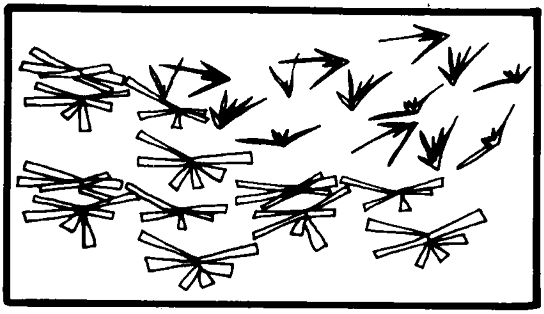


Plate 8 : Microcrystals of strychnine with Sodium phosphate

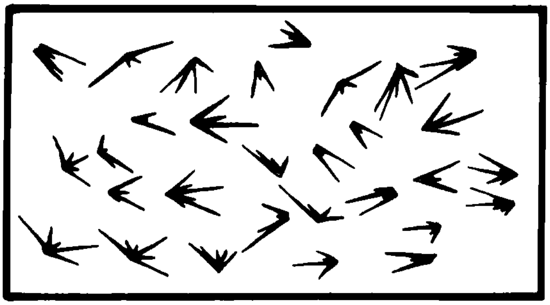


Plate 9 : Microcrystals of strychnine with Potassium chromate

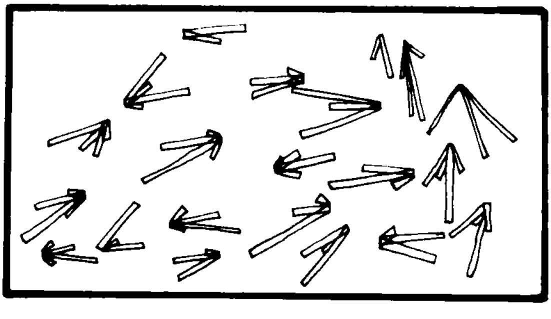


Plate 10 : Microcrystals of strychnine with Potassium Iodide

$CHCl_3$ into another separator containing 10 ml N H_2SO_4, shake, reject the $CHCl_3$ and add the acid washings to the first separator. Render alkaline with ammonia and extract the liberated alkaloids completely with successive portions of $CHCl_3$ (3x20 ml). Wash the $CHCl_3$ extract with 5 ml H_2O, dehydrate over anhydrous Na_2SO_4 and distill the $CHCl_3$ completely. Dissolve the residue in 15 ml of 3% w/v H_2SO_4, warm if necessary, cool, then add 2 ml concd. HNO_3 and few crystals of $NaNO_2$, allow to stand for 30 min. at ordinary temperature. Transfer the contents to a separator containing 30 ml of 10% NaOH, rinse the container with 2 successive portions each of 5 ml H_2O, add the rinsing to the separator and shake for about 2 min. Extract strychnine with $CHCl_3$ (3x10 ml). Wash the combined $CHCl_3$ extract with 2x10 ml H_2O. Dehydrate the $CHCl_3$ over anhydrous Na_2SO_4. Evaporate the $CHCl_3$, add to the residue 5 ml neutral 95% alcohol, evaporate and leave the residue on a boiling water bath for further 15 min. Dissolve the residue in about 2 ml $CHCl_3$, add 20 ml N/20 H_2SO_4, evaporate $CHCl_3$ completely, then titrate excess acid with N/20 NaOH using methyl red as indicator. Each 1 ml N/20 H_2SO_4 $\equiv$ 0.01672 g strychnine. The result is multiplied by 1.02 to correct for the loss of strychnine during oxidative destruction of brucine.

Another method based on the same principle was also reported (68).

A method for simultaneous determination of strychnine and brucine was presented (69). A suitable vol. of an aq. soln. of the 2 alkaloids, made alk. with NaOH, is extracted first with Et_2O, then with $CHCl_3$, and the combined extracts, dried with anhydrous Na_2SO_4, is filtered into a tared flask. After evaporating the solution, the combined weight of brucine and strychnine is determined, following which the residue is dissolved in alcohol and H_2O and titrated with 0.01 N HCl.

Another method (70) for determination of micro amounts of strychnine using heteropoly acids was described.

Titrate 20 ml of 0.001 - 0.002 M solution of strychnine, containing 1 ml of 1 M HCl (or 2 ml of M NaOAC) with 0.00125 - 0.005 M tungstosilicic, tungstophosphoric or molybdophosphoric

acids. The molar ratios of the stoichiometric compounds formed in pH 1 (HCl) or pH 7 (NaOAC) solutions are respectively : with tungstosilicic acid 4, 4; with tungstophosphoric and molybdophosphoric acids 3, – .

A method for determination of strychnine nitrate by titration with silicotungstic acid was also reported (71).

Another method (72) for rapid titrimetric determination of strychnine with ± 1% accuracy is based on addition of $CHCl_3$ and a buffer solution to the base dissolved at pH 2.8. Titrate with Na dioctylsulfosuccinate (dimethyl yellow screened with oracet blue as indicator). The color change is from green to pink.

Satisfactory results were obtained for the determination of strychnine nitrate by using K-Bi complex; the liberated EDTA is titrated with 0.01 M $ZnSO_4$ with the Na_3BO_3 buffer (73).

A direct amperometric titration of amines with sodium tetraphenylborate (Na B Ph_4) solution, based upon anodic depolarization at a dropping Hg electrode (74), was found suitable for determination of strychnine.

A micro-procedure, permitting determination of strychnine in mg rang was described (75). A small separatory funnel ($\leq$ 50 ml capacity) is used to shake out the sample with 2 ml of 10% NH_4OH, then with $CHCl_3$. The $CHCl_3$ extract is transferred to a second small separatory funnel, washed with H_2O, placed in a 50 ml (or less) Erlenmeyer, dried, treated with 0.5 ml of 95% alcohol and with 2.0 ml of 0.01 N H_2SO_4, heated gently to aid solution and the excess acid titrated against 0.01 N NaOH from a 2 ml microburet, using 1 drop of methyl red solution as indicator. Good accuracy was obtained with strychnine - HCl and a mixture of strychnine salt with Na methyl arsenate.

The use of 2, 5-dimethylbenzene sulfonic, 3, 4-dichlorobenzene sulfonic and 4-(benzene sulfonyloxy) benzene sulfonic acids (76) and 3 methyl-6-nitrobenzene sulfonic acid, 2, 5-dichlorobenzene sulfonic acid (77) were used for titrimetric determination of strychnine in injection solutions, giving reproducible results and lower mean standard deviation.

Another method depends on the precipitation of strychnine from an acidified dilute solution with K_3 Cr $(SCN)6$. The excess reagent hydrolyzed in the filtrate by alkalinization and - SCN determined by titration with BrO_3^- The reagent/strychnine ratio in the ppt. was 1:3, relative deviation ± 0.3 - ± 0.7% (78).

Other method for titrimetric determination of strychnine at polarization potentials of up to 1000 mv was reported (79)

A more recent method for photometric titration of strychnine nitrate was described (80). The alkaloidal salt is mixed with 1 N HCl, diluted with H_2O, and titrated with tungstophosphoric acid with photometric end point indication.

7.3.2 Non-Aqueous Titration

The Egyptian Pharmacopoeia (1972) describes the following method: Dissolve 0.5 g strychnine hydrochloride in 30 ml acetic anhydride. Add 10 ml $HgCl_2$ and 20 ml dioxane. Using 2-3 drops of crystal violet reagent, titrate with 0.1 N $HClO_4$. Carry a blank experiment and calculate the mls of $HClO_4$ consumed; 1 ml 0.1 N $HClO_4$ ≡ 0.03343 g strychnine.

Another method based on titration of strychnine with 0.1 N of hydrochloric acid complex of chloroaluminium isoproxide in $CHCl_3$ (81). The deviation was ± 1 % in the range of 38-245 mg of alkaloid.

A third method for determination of strychnine nitrate injections in an anhydrous medium was presented (82). Adjust the pH of the injection solution to 8-9 with $NaHCO_3$. Extract 3 times with $CHCl_3$, filter the extract and titrate in the presence of dimethylyellow by 0.005 N p-toluenesulfonic acid in dioxane.

Alkaloids in tincture of nux vomica were determined in non aqueous media, by their liberation with NH_4OH, extraction with $CHCl_3$ and titration with 0.02 N $HClo_4$ in dioxane in the presence of methyl red in PhCl (83); 1 ml 0.02 N $HClO_4$ is equivalent to 6.688 mg alkaloids (strychnine + brucine).

Another method for non aqueous determination of strychnine was reported (84). The

following acids in 0.005 N dioxane solutions are used as titrants: naphthalene sulfonic, naphthalene-2-sulfonic, 5-nitro-naphthalene-6-sulfonic, and 2-propoxynaphthalene-6-sulfonic. The titrants contained 1% phOH and were standardized against atropine or brucine dissolved in $CHCl_3$ using 0.1% dimethyl yellow as indicator. For the determination, 5 ml of a solution to be analyzed were taken (containing about 5 mg alkaloidal salt). its pH adjusted to 8-9 with saturated $NaHCO_3$ solution or 5% NaOH, and then extracted 4-5 times with 5-10 ml $CHCl_3$ each. The combined extracts are filtered and titrated; the error was $<\pm$ 1%.

Titration in non aqueous media was also adopted for determination of strychnine and other alkaloids in elixir tonic and Cola extract (85). The preparations are extracted with $CHCl_3$ - EtOH (5:1), and the $CHCl_3$ phase is separated by TLC on silica gel DG plates using EtOH-$CHCl_3$ (88:12) for development. The strychnine spot was identified by sequential treatment with alcoholic KI, 25% HCl - 95% EtOH (1:1), and Dragendorff reagent. For quantitative determination, the drug is extracted with $CHCl_3$ - EtOH (20:3) and the $CHCl_3$ phase was mixed with AC_2O, diluted with C_6H_6 and titrated with 0.1 N $HClO_4$, using crystal violet indicator. The relative standard deviation is 3%.

7.3.3 Potentiometric Titrations

Strychnine among other alkaloids was investigated in the concentration range 10^{-6} to 10^{-2} M with potentiometric titration (86). An adsorption microelement Sb/gel/K Cl (0.1 N), Hg_2 Cl_2 /Hg connected with a lindemann quadrant electrometer was used. The gel layers contacting the indicating electrode with the reference one were made of acacia gum in 0.05 N KCl. The titration was carried out in petroleum ether, CCl_4, cyclohexane, C_6H_6, and acetone. As a titrant, Cl_3 – C CO_2H or picric acid in the same solvent as the titrated alkaloid was used. The experimental conditions are most favorable when the difference between the dielectric constants of the solvent and the gel adsorption layer is large.

Another method described the determination of strychnine nitrate potentiometrically by titration with 2.5% Na tetraphenylborate using valinomycin ion selective electrode (87).

A new electrode was developed which was sensitive and reasonably selective for strychnine (88). It is based on the use of the strychnine-picrolonate ion-pair complex in nitrobenzene as a liquid membrane. The electrode shows Nernstian response down to 10^{-5}M strychnine solution, over a pH range of 2-7 with a cationic slope of 57 mV/concn. decade from $5x10^{-2}$ to $5x10^{-5}$M. Direct poteniometric determination of samples down to 5 ppm shows an average recovery of 101.3% and a mean standard deviation of 2.4%. Poteniometric titration with either picrolonic acid or Na tetraphenylboron shows an average recovery of 99.1% and a mean standard deviation of 1.2%. No interferences were caused by other alkaloids and many of the substances normally occurring as natural contaminants with strychnine.

Another method (89) for the potentiometric determination of strychnine was presented. It is based on the formation of insoluble alkaloid picrate salt using a picrate ion-selective indicating electrode. The average error was about 4%. The method was successfully applied to pharmaceutical preparations.

Determination of strychnine by a selective liquid membrane electrode was also described (90). The electrode strychnine-tetra (m-methylphenyl) borate was prepared from strychnine and the borate compound. The calibration plot was linear in the range $1x10^{-2}$ to $5x10^{-6}$M, and the lowest detection limit was $1x10^{-6}$M. Optimal pH for the determination of strychnine at 10^{-5} to 10^{-2}M was 2-6.

7.4 Compleximetric Determinations

A method for the gravimetric determination of strychnine sulfate was described (91). The method based on conversion of the alkaloidal salt to the corresponding hexabromotellurate by reaction with hexabromotelluric acid.

A second method based on the same principle was described for strychnine analysis and control (92).

The strychnine sulfate is dissolved in HCl and precipitated as hexachlorotellurates with H_2 ($TeCl_6$). The ppt. is washed with HCl, dried at 50°C and weighed. The error of the gravimetric method was $\leq$ 0.75%.

Other methods for gravimetric and titrimetric determinations of strychnine are presented and discussed (93).

7.5 Spectrophotometric Methods

7.5.1 Colorimetry

A colorimetric procedure was described for the assay of strychnine nitrate in tablets containing codeine base and other ingredients (94). The assay based on the interaction of strychnine reducing products and HNO_2 yielding a measurable red color. The tablet alkaloids are Et_2O - extracted and strychnine is determined colorimetrically. The accuracy was 1%.

A second colorimetric method for determination of strychnine and brucine in their mixtures was presented (95). Strychnine was determined after the separation of brucine. Add H_2SO_4 (3%, 7.5 ml) to the mixture alkaloids ($\sim$ 1 mg), cool, add 1.5 ml of a mixture of HNO_3, d. 1.42 - H_2O (1:1), make the mixture alkaline with NaOH after 10 min, extract strychnine with $CHCl_3$, (3x10 ml), evaporate the $CHCl_3$ extract to dryness, add 4 ml H_2O, 6 ml HCl (d. 1.12), and 1 g Zn dust to the residue, heat the mixture on a boiling water bath for 20 min. and cool. Add $NaNO_2$ (10%, 1 drop) to the mixture, dilute to 50 ml and measure the optical density of the solution on a photoelectric colorimeter using a green filter. The error of the method was ± 0.76%.

A third method for separation and colorimetric estimation of strychnine, among other stimulants, in doping horse urine was reported (96). Strychnine is separated from other ingredients by TLC on silica gel G plates using MeOH as a developer. The corresponding spot is eluted and determined colorimetrically. 18% of the administered strychnine, was detected in the urine of the doped horses within 48 h after administration.

A fourth method was described for colorimetric estimation of strychnine in biological preparations (97). Strychnine is separated by ascending paper chromatography by using BuOH-ACOH-H_2O (4:1:5) as a solvent and BiI_3 as a developer. The alkaloid in the spots is separated by acidifying with 0.1 N HCl and determined colorimetrically with bromothymol blue. After investigating 13 lethal cases of poisoning, it was found that in organs preserved with HCO_2H, the amounts of strychnine are far lower than in the same organs which were not preserved.

A colorimetric method for determination of the sum of strychnine and brucine in seeds and galenic preparations of nux vomica was discussed (98). A 0.01 g sample of finely ground seeds are shaken, 30 min, with 5 g $CHCl_3$, 10 g Et_2O, and 2 ml 10% Na_2CO_3. The organic solvent evaporated to dryness, the residue dissolved in phosphate buffer, pH 5.5, to make 100 ml. A 1 ml aliquot shaken 5 min with 20 ml C_6H_6, 2 ml phosphate buffer, pH 5.5, and 3 ml saturated solution of bromothymol blue. The developed color in the organic layer is measured colorimetrically. The same procedure was used for determination of the alkaloids in dry extracts and tinctures prepared from nux vomica.

Two other methods for colorimetric determination of strychnine were developed; one (99) was a modification for the other (100). The modification resulted in increasing the sensitivity of the assay. Evaporate 10 ml nux vomica tincture or 1 ml of its extract to dryness. Extract residue with 1% aqueous H_2SO_4. Transfer the aqueous extract into separatory funnel through filter paper and wash with 2 ml dil H_2SO_4. Add aqueous washings to mother liquor and render alkaline to litmus with 10% NH_4OH solution (Ca 5 ml). Shake with successive portions of $CHCl_3$ until alkaloids are completely extracted (3x20 ml). Evaporate combined $CHCl_3$ extract and dilute to 25 ml with the same solvent. Take 0.5 ml aliquots for the proposed colorimetric method as follows: Evaporate the $CHCl_3$ solution to dryness on a water bath, add 5 ml HCl and 1 g granulated zinc, heat 10 min in a boiling water bath or let stand 20 min at room temperature, and then dilute with 5 ml distilled

water. Cool to room temperature, transfer quantitatively to 25 ml volumetric flask, rinse container several times with small portions of distilled water, and dilute to volume with distilled water. To different aliquots of the reduced solution (containing $\sim 100\,\mu g$ strychnine) add 3 ml nitroprusside-acetyldehyde reagent (10% acetaldehyde in 1% sodium nitroprusside) and adjust the volume to 12 ml with distilled water. Let mixture stand 15 min and measure color at 530 nm against blank of 9 ml water and 3 ml nitroprunide-acetaldehyde reagent. The percentage was calculated from a standard curve of strychnine. The developed color obeyed Beer's law in the range of 10-320 μg/12 ml. The standard deviation was 1.02.

A fast identification and determination method for strychnine in heroin specimen was reported (101). It depends on measuring the pink color developed after boiling with $HgCl_2$ and Zn and addition of $NaNo_2$.

Two spectrophotometric procedures depending on complexation of strychnine with hexabromotelluric acid (91) or hexachlorotelluric acid (92) were presented. The ppt. is dissolved in MeOH and strychnine determined photometrically at 450 nm for the first complex and at 420 nm for the second complex. The error of the second process was $\leq$ 1.6%.

A method for the extractive spectrophotometric determination of strychnine with Solochrome Green V150 was presented (102). The method depends on extracting strychnine 1:2 ion pair with Solochrome Green V150 into $CHCl_3$. The absorbance of the organic phase is measured at 520 nm (molar absorptivity = 3.7200×10^4). Beer's law was obeyed for 1-300 μg strychnine; the error is ± 1.5%.

The differential acid dye method for determination of different pharmaceutical compounds was successfully applied for the determination of strychnine (103). The method involves extraction of a mixture of 5 ml pH 4.2 K H phthalate buffer, 5 ml Methyl orange, and 3 ml of the alkaloidal solution of a suitable concentration, with $CHCl_3$, followed by absorbance measurement of the pooled extract against a reference extract. It was reported that the results obtained

by traditional and differential acid dye method were comparable and the reproducibility of the latter method was higher.

A method for spectrophotometric determination of strychnine nitrate and other alkaloids was presented (104). Strychnine nitrate is dissolved in acetate buffer pH 4.1-4.2, Chromazurol S is added, after 30 min the reaction mixture is extracted with $CHCl_3$ and optical density is measured at 510 nm. Relative error of the determination was ± 1-3%. Chromazurol S reacted also with 34 other alkaloids.

A spectrophotometric titration method was described for the determination of some alkaloids and their dosage forms using 0.005 M chloranilic acid solution in 1, 4 dioxane as the titrant (105). The end point is determined by measuring the change in absorbance of the sample at 535 nm. Quantitative recoveries with good reproducibility were reported for strychnine.

Comparative study on various sulfonic azodyes for alkaloids analysis (106) showed that Litol red was the most suitable for colorimetric determination of strychnine by ion-pair extraction in $CHCl_3$.

Seven azo dyes including orange II were evaluated for the spectrophotometric determination of alkaloids and compared with the two most common dyes for such determinations, methyl orange (II) and bromothymol blue (107). Orange II was more sensitive for strychnine determination in blood and urine and tolerated greater amounts of extraneous ions (such as Mg^{2+}, Co^{2+}, Mn^{2+}, F^-, $C_2O_4^{2-}$).

Another method for colorimetric determination of strychnine with chloranilic acid was described (108): in dioxane - $CHCl_3$ medium, the acceptor chloranilic acid forms purple 1:1 mol. complexes with the alkaloid which gave maximum absorption at 535 nm.

Strychnine was determined in 4 pill and powder preparations by TLC-Colorimetry (109). As an example, a pill preparation is powdered, extracted with $CHCl_3$-EtOH (10:1) and NH_4OH and the extract is chromatographed on a silica gel G plate (toluene-acetone-EtOH-NH_4OH, 8:6:0.5:2 as eluent). Spots are visualized with iodine

vapor and extracted to give a fraction, which is treated with bromothymol blue and benzene. The organic phase treated with KOH-EtOH is analyzed at 610 nm for the determination of strychnine. The calibration plot was linear up to 40 μg and the recovery was 89.84 - 93.96%; relative standard deviations were 2.59-4.80%.

A method for semiquantitative determination of strychnine in 10-50 γ quantities by the Weiszring oven colorimetric procedure was proposed (110).

Another colorimetric determination of strychnine was reported. It is based on the development of intensely colored radical ions due to the reaction of the alkaloid with selected polyhaloquinone and polycyanoquinane π acceptor (111).

7.5.2 Ultraviolet Spectrophotometry (UV)

The B.P. (1973, 1980) (112,113) describe a method of assaying strychnine in nux vomica seeds and galenic formulations as follows: Mix 0.4 g, in fine powder, with 2 ml of alcohol (70%). Add 5 ml of dilute ammonia solution and transfer with the aid of 25 ml of H_2O and 20 ml of $CHCl_3$ to a 60-ml ground glass, downward displacement, liquid-liquid extractor containing 60 ml of $CHCl_3$, the extractor being equipped with a distributor of four baffle discs and a 100-ml short-necked flask. Ensure that all lumps are broken down and that no powder adheres to the top of the distributor. Attach a reflux condenser and extract for 4 h with occasional swirling of the contents of the extractor. Transfer the $CHCl_3$ in the flask to a separating funnel and extract with 4 quantities, each of 20 ml, of 0.5M H_2SO_4. Wash the combined acid extracts with 10 ml of $CHCl_3$. Filter the aqueous solution through a small wet plug of cotton wool into a shallow dish and warm gently with stirring to remove traces of $CHCl_3$. Cool and dilute to 100 ml with N H_2SO_4. Dilute 20 ml to 100 ml with N H_2SO_4 and measure the extinction of a 1-cm layer at 262 nm and 300 nm. Calculate the % of strychnine from the expression 5(0.321 a - 0.467 b)/w, where a is the extinction at 262 nm, b is the extinction at

300 nm, and w is the weight of the powder taken.

For the assay of nux vomica liquid extract

The method mentioned for the powder was adopted, extracting 2.0 ml of the liquid extract, omitting the 2 ml of alcohol 70%, and taking 5 ml of the acid extractive for the preparation of the final solution. Calculate the % w/v of strychnine from the expression : 20(0.321 a - 0.467 b)/v, where a is the extinction at 262 nm, b is the extinction at 300 nm, and v is the volume of liquid extract.

For nux vomica tincture carry out the assay as described under the powder, extracting 5.0 ml of the tincture and omitting the 2 ml of alcohol 70%. Calculate the percentage w/v of strychnine from the expression : 5(0.321 a - 0.467 b)/v, where a is the extinction at 262 nm, b is the extinction at 300 nm, and v is the volume of the tincture.

A second method making use of the absorbance at the aforementioned wave lengths was presented (114). It depends on complexation of both strychnine and brucine with methyl orange at pH 5.0, and after treatment with 0.1 N NaOH, the liberated alkaloids are determined spectrophotometrically. The method was carried out as follows : Dilute 10 ml tincture nux vomica (0.125% w/v) to 100 ml with McIlvaine's buffer, (prepared by addition of 48.50 ml 0.1 M citric acid + 51.50 ml 0.2 M $Na_2HPO_4 . 2H_2O$ and adjusting pH to 5 with a suitable pH meter). Accurately transfer 2 ml diluted sample to 50 ml separatory funnel containing 10 ml dye solution in buffer. Shake 1 min with 10 ml $CHCl_3$; let layers separate completely. Transfer $CHCl_3$ layer to another 100 ml separatory funnel containing 10 ml 0.1 N NaOH. Extract aqueous layer 3 times each with 10 ml $CHCl_3$. Shake combined $CHCl_3$ extracts 15 sec to liberate the dye. Transfer $CHCl_3$ layer to 100 ml separatory funnel containing 10 ml 0.1 N H_2SO_4. Shake, transfer acid extract of the alkaloids to 25 ml volumetric flask, and dilute to volume. Measure absorbance of 1 cm pathlength at 262 and 300 nm. Calculate concentration (mg %) of strychnine, C_a, and brucine, C_b in the tincture from the following equation:

$$C_a = [(0.200\ A_1 - 0.290\ A_2)/0.5955] \times (1/4) \times (100/2) \times (10)$$

$$C_b = [(0.305\ A_2 - 0.005\ A_1)/0.05955] \times (1/4) \times (100/2) \times (10)$$

where A_1 and A_2 are absorbances of final solution; 0.305 and 0.005 are absorbances of 1 mg % strychnine; 0.290 and 0.200 are absorbances of 1 mg % of brucine, at 262 and 300 nm, respectively. The mean percentage recovery was 100.7±0.93 for strychnine.

Different other spectrophotometric procedures have been reported for the UV spectrophotometric determination of strychnine in crude drugs or formulations.

An old method makes use of the absorption band at 254 nm shown by strychnine in absolute alcohol (brucine shows maxima at 264 nm) (115). The alkaloid extract, containing both strychnine and brucine, is treated with 0.5 g of potassium persulfate in the presence of 3% H_2SO_4 for 1 h, thereby brucine is destroyed while strychnine is not. The soln.is then made alkaline and the strychnine extracted with $CHCl_3$. The residue left after evaporation of $CHCl_3$ is dissolved in absolute alc. and the extinction (E) at 254 nm of the soln.is measured. This E value is divided by E 1% at 254 nm (which is equal to 390) gives the concentration, as % of strychnine in the alcoholic soln. being measured.
Constituents which may absorb at 254 nm, if not properly removed would of course interfere with accurate quantitative estimation.

An ammoniacal suspension of the drug is extracted with $CHCl_3$ in a downward displacement liquid-liquid extractor. Strychnine and brucine are then extracted from the $CHCl_3$ with N H_2SO_4 and the strychnine determined spectrophotometrically (116). It was reported that the obtained results are in agreement with the official methods but saving half the determination time.

The two wave length procedure of analysis was also applied for determination of mixtures of strychnine and brucine at 252 and 262 nm (117) and at 255 and 264 nm in nux vomica tinture (118). The latter method involves

purification by adsorption on alumina and by cation exchange resin.

A method for the quantitative determination of strychnine in brucine or brucine sulfate was described (119). Brucine is nitrated and strychnine is extracted with $CHCl_3$. The $CHCl_3$ is then evaporated and strychnine is determined spectrophotometrically by dissolving the residue in 0.1 N H_2SO_4 and measuring absorbances at 254 and 275.5 nm. The difference between these absorbances, compared with similar differences at these wave lengths for standard strychnine solutions is a measure of the strychnine content; standard deviation was less than 0.03%.

A combined chromatographic and spectrophotometric method for determination of strychnine tincture was described (120). After paper chromatography and development with the Dragendorff reagent, the spot is extracted from a parallel undeveloped chromatogram with 0.1 N HCl and the amounts of strychnine present determined using the absorption of light at 300 nm.

An ultraviolet determination of strychnine in commercial bait formulations was developed (121). The strychnine is extracted in 0.5% (vol.) H_2SO_4 and the resulting extract is cleaned up. Strychnine is determined by the difference in absorbance at 254 and 287 nm. It was reported that the results obtained by this method correlate well with those obtained by a gravimetric determination of strychnine as the picrate.

Spectrophotometric determination of strychnine and brucine (100-500 μg) from acidic solutions was carried out after precipetation with the Mayer-Valser reagent. The ppt. alkaloid - HI + HgI is dissolved in 3 M AcOH and the alkaloid determined indirectly over Hg-dithizon (122).

UV spectrophotometry was also used to follow the decomposition of certain alkaloids, including strychnine, (123), on sterilization of their 1% aqueous solutions by autoclaving 20 min and 2,5,7 and 10 h at 120°. Two-dimensional thin layer chromatography is used as a reference check. UV spectrophotometric determination was accurate with samples heated up

to 2 h ; prolonged heating resulted in the formation of ppts. interferring with the determination.

Strychnine was determined photometrically at 365 nm by reaction with I_2 in $C_2H_4Cl_2$ (124). The results are reproducible and accurate.

A method for the quantitation of strychnine depends on the spectroscopic characteristics of the interaction of alkaloids with picrolonic acid in solvent of low and intermediate polarity was stated (125). The presence of alkaloids caused a red shift of the 322 nm band of the nonionized picrolonic acid to 355 nm, corresponding to the anionic resonance band. Considerable increase in absorbtivity which was dependent on both the basicity and molar concentration of alkaloids present was used for the determination of various alkaloidal salts including strychnine sulfate. The method was sensitive to 2μg/ml with accuracy of ±1.5% and standard deviation ±1.05-1.31.

Quantitative determination of strychnine nitrate and codeine in pills containing excipients of plant origin was reported (126). It depends on extraction of the alkaloid with $CHCl_3$, purification by passing through Al_2O_3 column and re-extraction of strychnine from $CHCl_3$ into 0.25% H_2SO_4. Chromatography on silica gel plates using $CHCl_3$ - acetone - NH_3 (12:24:1) was the final step in purification of the alkaloid. The suitable wave length for determination of strychnine was 254 nm. Strychnine nitrate was determined in capsules with $\leqslant$ ±1.52% error by UV spectrophotometry in 0.1 N H_2SO_4 (127).

Using the same principle, a spectrophotometric determination of strychnine nitrate in pills containing other ingredients such as phenobarbital, bromcamphor, Ca glycero-phosphate, and As_2O_3 was proposed (128). To 15 ml 0.1 N H_2SO_4 is added powdered pill containing $\sim$0.002g strychnine nitrate, followed by 30 ml Et_2O saturated with 0.1 N H_2SO_4 and extracted for 3 min The aqueous phase is reextracted, the extracts are washed with 10 ml 0.1 N H_2SO_4 after combining, filtered, brought to a desired volume and the absorbance determined at 254 nm. The proposed procedure eliminates phenobarbital and bromcamphor interference.

An improved spectrophotometric determination of teriary amine drugs, including strychnine, using malonic acid/acetic anhydride reagent was reported (129). The method involves the condensation of malonic acid and AC_2O under the catalytic effect of the tertiary amine function, either free or in the form of salt. The condensation product has 2 prominent absorption peaks at 333 and 400 nm. The apparent molar absorptivity of the 333 nm band was in the range of $1.37x10^5$ to $5.5x10^5$. Absorbance vs. concn. was linear up to 1.2 μg/ml of all drugs studied and the sensitivity was 0.1 μg/ml. The method was reported to be highly sensitive and specific for tertiary amine drugs without interference from primary and secondary amines or quaternary ammonium salt but does not distinguish between tertiary amines.

A method for determination of strychnine in nux vomica preparations, involving modification of the French Pharmacopoeia method was discussed (130). The modifications proposed are: maceration of the nut powder for 30 min in dilute ammoniacal solution; extraction with $CHCl_3$ - dil. NH_4OH for 2 h; evaporating the $CHCl_3$ extract to dryness, dissolving the residue in MeOH and examining the solution directly with UV spectrometry.

Strychnine was assayed in the seeds of *strychnos pierriama* by dual - wavelength spectroscopy (131) as follows: The seeds are dried, powdered and the powder ($\sim$0.4g) is treated with 2 N NaOH (1 ml) and mixed with $\sim$80 ml $CHCl_3$. The mixture is refluxed for $\sim$1 h. cooled, filtered and the filtrate is evaporated, treated with 1 N H_2SO_4 on water bath and diluted with 1 N H_2SO_4 to 100 ml. A 5-ml solution diluted with distilled H_2O (to 25 ml) and analyzed by dual-wavelength spectrophotometry at 255 and 278 nm for the determination of strychnine (relative standard deviation = 1.1%, recoveries were 98.9%).

First derivative spectrophotometric determination of strychnine and brucine in nux vomica liquid extract was reported (132). Strychnine and brucine are extracted from nux vomica extract, diluted with H_2O and NH_4OH by using $CHCl_3$ and are then back-extracted with 0.1 N H_2SO_4.

The 1st derivative spectra are measured at 262.8 nm for strychnine. The limits of detection were 3 μg/ml and the mean recoveries were 101.1%. They reported that this method was much faster than the British Pharmacopoea method.

Other methods for spectrophotometric assay of strychnine in different formulations and biological fluids and tissues were also discussed (133-137).

7.5.3 Infrared Spectrophotometry (IR)

Small amounts of strychnine and other organic basis, in urine could be identified by IR (138). The basis are extracted from urine with $CHCl_3$, transferred to spots of dil. H_2SO_4 supported on strips of Whatman 3 MM paper and chromatographed, after neutralization of the acid spot, using either BuOH-HCl or ISo-Bu Me Ketone - ACOH as solvent. Detection of the alkaloid is achieved by an iodoplatinate spray, or a bromocresol green spray. Strychnine may then be recovered for spectrophotometry by excising the spot, making alkaline with NH_3 and extracting with $CHCl_3$. Spectrophotometry is best carried out in a solution in an organic solvent in microcells with NaCl windows.

Another method involves the identification and estimation of strychnine by IR spectrometry after its extraction and separation by TLC on silica gel (139). The chromatogram eluates are concentrated on KBr micropellets. Spots containing about 10 γ of strychnine could be estimated with a precision of 8-15%.

7.5.4 Nuclear Magnetic Resonance: NMR

Strychnine was determined in nux vomica powder using NMR (140). The four aromatic protons of strychnine exhibit signals at 7.20 (protons 9,10,11), and at 8.20 ppm (proton 12), the latter being influenced by the carbonyl group of the lactam. The 2 aromatic protons of brucine form well defined signals at 6.73 (proton 9), and 7.88 ppm (proton 12), the latter also influenced by the lactam carbonyl. The height to the strychnine peak at 7.17 ppm, over the height to the brucine peak at 6.73 ppm, is a function of the percent strychnine in

the sample. The NMR method was checked by volumetric analysis of the mixtures.

7.5.5 Atomic Absorption

An indirect determination of alkaloids by atomic absorption spectrometry was applied for determination of strychnine (141). Strychnine is reacted with Reinecke salt solution, $PhNO_2$ is added and the mixture is shaken for 2 min. The $PhNO_2$ layer is separated and dehydrated and the Cr in the Ph NO_2 solution is measured by atomic absorption spectrometry. Cu^{2+}, Co^{2+} Mg^{2+}, Ca^{2+}, Na^+, Ni^{2+}, K^+, Pb^{2+}, Cd^{2+}, Fe^{3+}, NH_4^+, Cl^-, PO_4^{3-}, Br^-, NO_3^-, $Na_2S_2O_3$, monosodium glutamate, starch, and sucrose did not interefere.

7.6 Chromatographic Methods

7.6.1 Paper Chromatography

Results of separation of indole alkaloids including strychnine by paper chromatography (Whatman No. 1) in various solvents, were given (142). Petroleum ether saturated with formamide-benzene-EtOH (100:25:25), BuOH-HCl (conc.)-H_2O (50:7.5:17.5), BuOH-AcOH-H_2O (4:1:5) were suitable. Impregnating the paper with formamide + 8% NH_4 formate gave round spots.

Using Whatman No. 1, buffered by dipping in a 5% solution of sodium dihydrogen citrate, blotting, and drying at 25°C for 1 h, a solvent system was given for the detection of organic basis (143,144). For strychnine, a solvent composed of 4.8 g of citric acid in a mixture of 130 ml of H_2O and 870 ml of n. butanol is used; its R_f = 0.30 using UV and iodoplatinate spray reagent for location.

A method for quantitative separation of strychnine by paper chromatography on 'segment strip' and its colorimetric evaluation by adduct formation with dyes was discussed (145).

7.6.2 Thin Layer Chromatography (TLC)

The solvent system, (strong NH_3 : MeOH, 1.5:100), used for general screening of nitrogenous bases,was used for detection of strychnine on silica gel G layers (R_f=0.22) (146). Location was under UV and with acidified iodoplatinate spray reagent. TLC using Dragendorff's or the previous reagent was also reported for

separation and detection of nux vomica alkaloids (147,148).

A method for the identification of strychnine in biological material, using TLC was presented (149). The alkaloid is extracted with 0.02 N H_2SO_4 at pH 2.5-3.0. The extract is alkalinizėd with 20% NaOH to pH 8-9, extracted with $CHCl_3$ and chromatographed on silica gel KSK layers using $CHCl_3$ - Me_2CO - NH_3 (30:30:2) for development.

The use of TLC layers containing adsorbents, binders, fluorescent substances and colloidal silica for separation and identification of strychnine was discussed (150). C_6H_6-$CHCl_3$-Et_2NH (9:4:1) was the developer and spots were visualized under UV.

Application of different chromatographic techniques (TLC, GLC and HPLC) for rapid detection of drugs, intoxicants and related compounds, including strychnine was investigated (151).

An improved TLC method for detection and identification of basic drugs extracted from tissues was reported (152). This was achieved by a double development technique using CH_2Cl_2 : Me Et ketone: NH_3 (90:10:1.5 and 30:70:2). Identification was achieved after the traditional detection spray sequence with successive topical applications of : Marquis reagent. Mandelin reagent and a special NH_4 molybdate fuming H_2SO_4 reagent.

TLC was also applied for the detection and determination of strychnine and brucine (153). The two alkaloids are first separated by TLC and then determined photometrically with 0.5% picric acid in HOAC.

Another method for the determination of strychnine in Chinese medicines by dual wave length TLC-densitometry was also reported (154).

7.6.3 Adsorption Chromatography

A method for separation of strychnine from nux vomica seeds by adsorption chromatography was described (155). A 2 g sample of powdered seed material, made alkaline, is extracted with $CHCl_3$ and the solvent evaporated to dryness. The residue is dissolved in trichloroethylene and chromatographed on alumina (10g) column.

Strychnine is then eluted with 70 ml of CCl_4 containing 9% acetone. The solvent is then removed by distillation and the alkaloidal residue titrated with a standard acid.

7.6.4 Ion Exchange Chromatography

A procedure was described for separation of strychnine from nux vomica using ion exchange resin followed by its titrimetric determination (156). 0.3 g sample of the defatted powdered nux vomica seed material is extracted with a mixture of 5 g $CHCl_3$, 15 g ether and 1 ml of 10% ammonia. The $CHCl_3/Et_2O$ extract is then shaken for 30 seconds with 5 ml H_2O and the solvent layer is then evaporated to almost dryness, 1 ml of alcohol added, and evaporation is carried out to dryness. To the residue is then added 3 drops of 2% H_2SO_4, followed by 10 ml of 90% alcohol and the solution is passed through ion exchange resin (Amberlite IR-4B) to remove impurities, and the eluate is titrated with 0.01 N HCl, using the antimony electrode for electrometric titration, and the quantity of strychnine calculated.

Another method for the separation of strychnine from extracts of animal tissues using cation-exchange resins was reported (157). Liver tissue (100 g) is macerated in 500 ml of H_2O, and 100 ml 0.01N HCl is added. The mixture is heated to boiling and held in a boiling water bath for 1 h; then the mixture is diluted to 1 l with H_2O and filtered. The filtrate is put through a column of Dowex 50W x 12 (200-400 mesh) cation-exchange resin. Strychnine is eluted with 8 N HCl after removal of other alkaloids and the eluate is neutralized by $NaHCO_3$ before its quantitative determination.

A third method involves the use of a cation exchange paper for determination of strychnine was described (158). Although simpler than the colorimetric methods, but it needs a larger amounts of substance for analysis.

In a fourth article, strip of Amberlite SA-2(RH-form) and Amberlite SB-2(R OH form) are used for determination of strychnine (159).

A fifth method described the use of synthetic ion exchange resin for compleximetric determination of strychnine in pharmaceuticals (160). The solution containing alkaloids are passed through column packed with Wofatite KPS saturated with Zn^{2+} and Zn^{2+} is determined in the eluate by titration with edetic acid.

7.6.5 Gas Liquid Chromatography (GLC)

Although non volatile, numerous methods for GLC analysis of strychnine were reported. A system using 1% SE-30 on 100-120 mesh Anakrom ABS 6 ft x 4 mm I.D. borosilicate glass columns was described for GLC analysis of strychnine (161). The following operating conditions were adopted : column temperature, 250°C; carrier gas, argon; flow rate, 80 ml/min; detector, argon ionization or FID (H_2, 50 ml/min; air, 300 ml/min). The relative retention time of strychnine (relate. to codeine) is 4.83.

A method involving the use of glass columns (3 ft x 0.07 in) packed with Gas Chrom. P was described (162), for separation of different alkaloids including strychnine. The packing material is washed with concd. HCl, dried, treated with hexamethyldisilizane and coated with 1% of cyanosilicone, a polyester methyl silicone copolymer and cyclohexane dimethanol succinate polyester.

A third method for GLC determination of drugs, including strychnine in body fluids was reported (163). Four columns are used (2.5% Marlophene 87 (M 87), 1% Neopentylglycol sebacate (NGSB), 2.5% Silicon Rubber SE 52 and 1% Versamid 900 (SE 52/V 900). N_2 is used as a carrier gas and Me myristate, Me stearate and Me behenate are used as standards. Best results are obtained with a low temperature, a lightly packed column, and a sensitive detector.

A fourth method which allows GLC detection and quantitative determination of strychnine in brucine and vice versa, and allowed separation of the alkaloid bases in extracts of *Strychnos nux vomica* and *S. icaja* was presented (164) A FID, used for detection, N_2, as carrier gas (35 ml/min), and a 2-ft stainless steel column 1/8 in diameter, packed with Aeropak

30 (100-200 mesh) impregnated with 5% SE-52 as stationary phase. To determine strychnine in brucine, dissolve 100 mg of the sample in a mixture of 5 ml 5% H_2SO_4 and 15 ml H_2O. Heat if necessary. After cooling to room temperature, added 20 ml 10% NaOH and extract with successive 20 ml portions of $CHCl_3$. Filter the combined $CHCl_3$ fraction. Add 5 ml 95% EtOH; and evaporate to dryness. Dissolve the residue in successive 10-ml portions 95% EtOH, and dilute to 100 ml with EtOH. Inject 0.4 μl solution into the gas chromatograph system described. Percent of strychnine = 84 A_1/A_1+A_2 where A_1 is the area of strychnine peak and A_2 is area of brucine peak. Retention time of strychnine was 5.85 min with column temperature, 250°C and injector temperature 280°C.

A fifth GC method described determination of strychnine in biological materials (165). Tissue homogenate at pH 8.6 is stirred with acetone for 15 min. The mixture is heated and the acetone volatized. The mixture is acidified to pH 3.3 with 5% C Cl_3CO_2H, stirred for 30 min., and filtered through sand. The filtrate is extracted with $CHCl_3$ which is concentrated and an aliquot is injected on a stainless steel column packed with 30% SE-30 on Chromosorb W at 250°. Recovery from liver is 90%.

Another method presented a GLC procedure for determination of strychnine is grain baits (166), using glass column (6 ft x $\frac{1}{4}$ in.) packed with 5% SE-30 on Chromosorb W (DMCS) and FID for detection. The grain baits containing strychnine are ground, mixed, and extracted by shaking with $CHCl_3$ containing 1,3,5-triphenylbenzene as internal standard. Without further cleanup, extract filtrates are injected directly in a gas chromatograph. Peak height ratios are used for quantitation of strychnine with 2 μg sensitivity and recoveries ranging from 89.9 to 91.7%.

The retention index I_R and Δ I_R were considered for the gas chromatographic assay of different compounds including strychnine (167).

Another method for GLC analysis of strychnine after its silylation with N-methyl-N-(trimethylsilyl) hepta fluorobutyramide I)- BuOAC

(40:1:59) was discussed (168). An alkali flame ionization detector (AFID), and temperature programming with 15°C/min from 150-260°C are adopted.

A gas chromatographic determination of basic drugs in urine or blood and its application for doping analysis was reported for strychnine (169).

A rapid gas chromatography method for the qualitative detection of basic drugs, including strychnine, in postmortem blood, using a nitrogen phosphorous detector was described (170). The method involves a 1-step extraction from 1.0 ml of postmortem blood buffered to pH 9.0-9.5 into n-Bu chloride. No back extraction or clean up steps are required. Extracts are injected on 3% OV-1 and 3% OV-17 columns coupled to NP detectors. Sensitivity limits are drug dependent and range from 200-500 ng/ml for the various drugs listed in the report.

Combined use of fused silica capillary columns and nitrogen-phosphorus detection for the toxicological analysis of illicit heroin samples was successfully applied for detection of nanogram amounts of strychnine (171). The method involves the use of capillary columns of CP-Sil 5 or CP-sil 8 fused silica, deactivated with a polysiloxane at high temperature and diacetylnalorphine as the internal standard. The same column was also used in the analysis of an extract of the small intestinal content in an overdose case (172).

Drug screening via gas chromatography, fused silica capillary columns coated with a 0.25μm film of SE 30, yielded reproducible retention indexes for strychnine (173). The screening was performed on a temperature programmed columns at 100°-295°C at 5°C/min with the carrier velocity of 45 cm/s at 100°C. The capillary column provides superior resolution and/or greatly reduced analytical time relative to packed columns and is thus well suited to emergency toxicological situations in forensic laboratories.

The various substances present in street samples of narcotics (including strychnine) were detected in a single analysis by means of silylation and high-resolution gas chromato-

graphy (174). This technique allows the detection of narcotics, of most common adultrants, and of organic diluents at the same time. The method is highly sensitive and has high resolving power.

The use of the combined technique GC/MS for identification of strychnine among other compounds extracted from biological materials in a veterinary toxicology laboratory was discussed (175).

Gas chromatography employing 3% OV-1 on chromosorb W-HP for rapid detection of drugs, intoxicants and related compounds, including strychnine, was also reported (151).

Another GLC method for separation of some strychnos alkaloids was discussed (176).

An experiment was carried out by us on the effect of silylation on GLC characters of strychnine. Under our operating conditions, the non derivatized base showed a retention time 6.3 min. Although the peak was fairly well defined and sharp, but its base line was spread out over a period of approximately 6 min. The silylated strychnine, on the other hand, showed a sharp well defined peak at 6.0 min retention time with a highly reduced baseline spread. The sensitivity of detection was much higher (Fig. 11).

The following operating conditions were adopted.

Column, 3% OV 1 on Gas Chrom. 80/100 mesh; temperature, isothermal at 250°C; detector FID H_2 flow, 16 ml/min; air flow, 160 ml/min); carrier gas, N_2 with a flow rate, 40 ml/min; injector and detector temperature, 250°C. Silylation was carried out by using Tri-Sil/BSA reagent and a small amount of pyridine as a solvent.

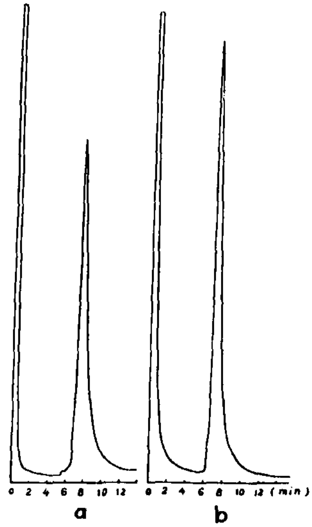
The apparatus used: Gas Chromatograph - Varian Model 3700 with Dual FID.

Fig. 11.

GLC of strychnine

(a) Free base

(b) Silylated base



7.6.6 High Performance Liquid Chromatography (HPLC)

HPLC offers obvious advantages by combining a separative power superior to that of TLC with a means of accurate quantitation and easy trapping of eluted material for subsequent confirmation of structure. This technique is moving fast for the analysis of many components. Because all the experimental work in HPLC is performed at ambient temperature, the problem of thermal instability is eliminated.

Using Corasil I with 1.1% Poly G 300 as a stationary phase and heptane - EtOH ranging from 100:5 to 100:10, v/v as mobile phase, quantities in nanogram level of strychnine can be satisfactorily analyzed with reasonable accuracy (177).

A paper describing the high-speed liquid chromatography of some alkaloids including strychnine, using liquid-solid chromatography was published (178). The wave length of detection was 255 nm. The column was a stainless-steel tube, 30 cm x 2 mm I.D., filled with Merckosorb Si 60 (5 μm). The column temperature was maintained at 20°. The retention time (RT) of strychnine in five different mobile phases and their flow rates (FR) are as

follows: $CHCl_3$ - MeOH (9:1), RT, 6.9, FR 0.81; $CHCl_3$ - MeOH (8:2), RT 3.9, FR 0.81; $CHCl_3$ - MeOH (7:3), RT 4.7, FR 0.75; Et_2O - MeOH (7:3) RT 9.4, FR 1.43; Et_2O - MeOH (6:4), RT, 7.3, FR 1.34.

Using the same column and as mobile phases, Et_2O containing 1% of diethylamine at a flow-rate of 2.0 ml/min at 205 kg/cm^2 and Et_2O - MeOH (1:1) at a flow-rate of 1.15 ml/min at 200 kg/cm^2, strychnine and other related alkaloids were analyzed (179). RT of strychnine was 7.2 and 12.4 min in the two systems respectively.

A simple and rapid HPLC procedure is described for the determination of strychnine in grain baits (180). The baits are ground, mixed, and extracted by shaking with $CHCl_3$. Without further cleanup, extract filtrates are injected directly into a liquid chromatograph. The analysis is completed within 7 min and peak heights are used for quantitation. Separations were made on a 30 cm x 4 mm I.D., stainless steel column packed with μ porasil (8-12μm silica). The eluting solvent was MeOH-$CHCl_3$ (10:90) at a flow rate of 2.0 ml/min. Recovery of spiked samples ranged from 91.5 to 95.2%. Confirmation of strychnine from a commercial sample was made by high resolution mass spectrometry with mass agreement to 1.2 ppm.

A procedure is described for the qualitative detection of strychnine among other contaminants in an illicit diamorphine seizures by means of high-pressure cation exchange chromatography (181). The illicit diamorphine samples are homogenised by grinding in a mortar and 20-25 mg, accurately weighed, are dissolved in 50% aqueous methanol. The solution is made up to 2 ml in a volumetric flask and 2μl are taken for an initial qualitative analysis using the following chromatographic conditions: column, 120 cm x 2.1 mm. Zipax SCX; eluents : (a) H_3BO_3 (0.2 M, aqueous), adjusted to pH 9.3 with 40% NaOH, (b) H_3BO_3 (0.2 M, aqueous) - acetonitrile-n-propanol (86:12:2) adjusted to pH 9.8 with NaOH (40% aqueous); flow rate, 2 ml/min; linear gradient, 0-100% (b) in 6 min; UV absorbance detector, 270 nm, 0.2 absorption units full scale. Strychnine was found in

many of the analyzed samples, retention time, 6.3 min and retention volume 12.8 ml (Fig. 12).

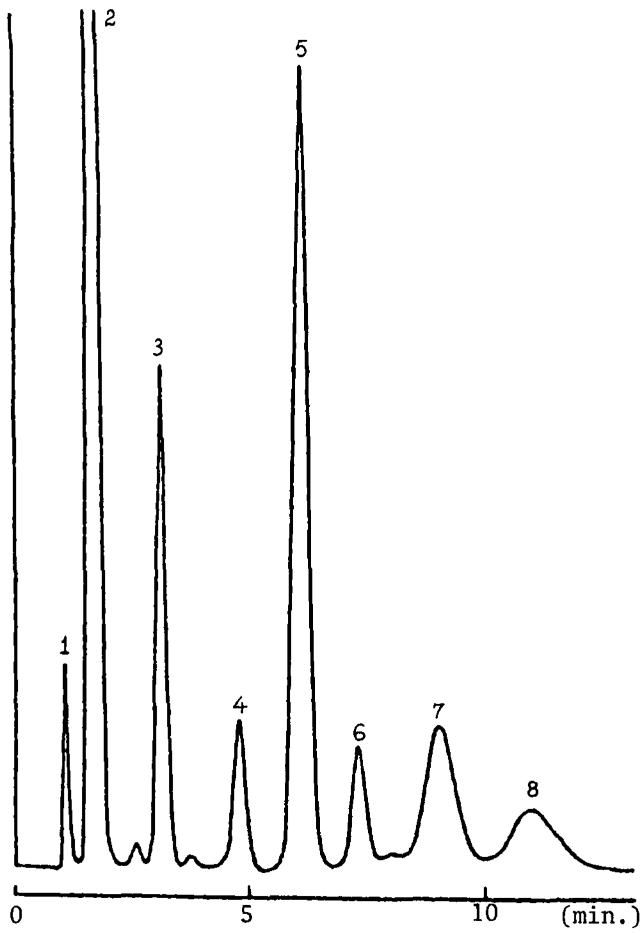


Fig. 12. HPLC of some constituents of illicit diamorphine seizures. For column conditions, see text.
1 = Barbitone; 2 = caffeine; 3 = morphine; 4 = monoacetylmorphine; 5 = strychnine; 6 = diamorphine; 7 = quinine; 8 = cocaine.

The retention of strychnine relative to morphine, when analyzed by HPLC among a wide range of drugs of abuse, was 1.57 (182). The following conditions were adopted: column, stainless steel, 25 cm x 4.6 mm I.D. filled with small size silica 'fines'; solvent, MeOH-2 N ammonia solution - 1 N ammonium nitrate solution (27:2:1); flow-rate, 1 ml/min; pressure, 1500 p.s.i, room temperature; detector, UV at w.l. 278 nm. A weighed amount of sample is made up in either water or dilute HCl to known concentration, usually 10 mg/ml, and dissolution is assisted by immersion for about 1 min in an ultrasonic bath. An aliquot of 1-5μl of this sample is injected on to the chromatographic column. Peak heights are used for quantitation.

A simple, rapid extraction and subsequent determination for strychnine, using HPLC with a reverse-phase solvent system was described (183). Stomach contents or grain bait containing strychnine are made alkaline with NaOH and extracted with $CHCl_3$. Extract filtrates are injected directly into a liquid chromatograph without further preparation, except for dilution, if necessary. Peaks are resolved within 3.5 min and peak heights are used for quantitation. HPLC of strychnine was carried out on a 30 cm x 4 mm I.D. stainless steel column packed with Bondapak C_{18}. The solvent program was 0.005 M phosphate buffer-MeOH (60 + 40) at a flow rate of 1.5 ml/min. Recovery from spiked stomach contents was 93.9%. The detection limits, using the 254 nm UV detector, was 5 mg. The strychnine peak was collected and subjected to TLC with standard for confirmation.

Reversed-phase HPLC employing octadecylsilanized columns (RP 18) was described for detection of strychnine (151).

An HPLC method was developed for the quantitation of strychnine in urine of children with nonketotic hyperglycinemia and other development disorders treated with the alkaloid (184). Mobile and stationary phases were polar, i.e. MeOH-H_2O-330 g/kg NH_3 ammonia (vols., 85

ml + 14.2 ml + 0.8 ml) and LiChrosorb Si-60, 7μm. Brucine was the internal standard. Extraction was performed by the Extrlut technique. At strychnine nitrate concentrations in urine of 21, 126 and 760μg/L, recovery was 92.1, 98.1 and 102.5. A child with nonketotic hyperglycinemia under continued strychnine treatment excreted 1-13.6% of the daily dose unmetabolized in urine. The method was also suitable for the estimation of unreacted strychnine in tissue extracts.

The determination of strychnine in seeds of *S. piersiana* or their preparations, included extraction with $CHCl_3$ - NH_4OH (9:1), separation of the organic phase, and injection of an aliquot into a HPLC column (4 mm diam. x 250 mm) containing silica gel (3-5 mμ, irregular) and Et_2O-MeOH-NH_4OH (80:20:1 v/v) as mobile phase (flow rate 2 ml/min.) for analysis. Detection was at 250 nm. The relative standard deviation was 1.29% (measured by the peak height method) and the recovery was 99.3%. The calibration plot was linear to 0.75μg. The method was simple and rapid, and may be used in routine analysis (185,186).

Measurement of strychine by HPLC in biological media was also reported (187). $CHCl_3$ at basic pH was used to extract strychnine and the internal standard quinine from plasma. HPLC was carried out on a silica gel column using as eluent concentrated NH_4OH in MeOH at a flow rate of 1.1 ml/min and detection at 254 nm. Recovery from plasma was 83%, the lower limit of sensitivity was 0.625μg/ml, and intra-and inter-assay variance was 3-5% and $<$ 10%, respectively.

A method for identification and determination of strychnine and brucine in nux vomica extracts and other pharmaceuticals using HPLC was described (188). The extracts were first purified by passing through a Sep Pak C_{18} - cartridge column and HPLC carried out on a reversed-phase ODS column with MeCN-H_2O (25:75) as the mobile phase containing 1-heptanesulfonic acid as the counterion. The recovery was $>$98.1.

Another method for rapid estimation of strychnine in tincture nux vomica BP was reported (189). The method was a modification of

a previous procedure (182). This involves the use of a shorter column (12.5 cm x 4.6 mm I.D.), changing the stationary phase to spherical particles (Hypersil), increasing the flow rate (2 ml/min) and changing the detector wave length from 278 to 254 nm; the net effect being to decrease the retention time of strychnine from 14 to 3.6 min and 6 min were sufficient to separate strychnine, brucine and the minor alkaloids of nux vomica. Concentrations were calculated using either peak areas or heights and comparing against an external standard (0.15% w/v strychnine base in 45% ethanol).

Other articles discussing the applications of different HPLC techniques for analysis of strychnine among drugs of forensic interest, in veterinary toxicology, in pharmaceutical formulations, in mixture with other basic drugs and for screening of drugs and insectides were also reported (190-194).

7.6.7 Electrophoresis

Electrophoretic determination of strychnine, brucine and quinine in tincture mixtures was developed (195). The separation was done in 2 N HCO_2H solution (pH 1.5) at 0.5 ma./cm and 10 v./cm. The spots were developed with Dragendorff reagent, and the concentration determined photometrically or by planimetry. Errors, were within 5-12%.

7.7 Radiometric Determinations

A radiometric titration method has been developed to enable microdetermination of many alkaloids, including strychnine (196) in the form of their salts (hydrochloride, phosphate or sulfate). The method was carried out as follows:

For hydrochlorides: Add a known volume of concd. HNO_3 to 0.5-3 ml of the alkaloidal salt in a centrifuge test tube, to result in 0.7-0.8 M HNO_3. Then titrate with 0.01 M $AgNO_3$ (approx. 3000 c.p.m./cc). After each addition of $AgNO_3$ solution, agitate to homogenize and centrifuge with complete sedimentation of the ppt., transfer a known volume of the clear solution to the γ-counter, and after counting, return to the test tube, and repeat sequence. It is necessary

to make at least 2 additions of titrating agent before the equivalent point and at least 3 additions after it. The equivalent point is determined graphically by extrapolation, with an error of ±0.4%. For phosphates : bring the alkaloidal salt with dil. NaOH to pH 8 and titrate with $AgNO_3$ as before; but stronger agitation and better centrifugation are required, the error being ±0.8%. For sulfates : titrate in a pH 7 alcoholic medium in which the Ag salt is insoluble, the error being 0.8%. The method can be adapted to mixtures of alkaloidal salts by titrating at different pH values.

Another method for rapid determination of strychnine with Mayer's reagent was based on radiometric titration (197). Mayer reagent was prepared by irradiation of 1.358 g $HgCl_2$ in a neutron flux of 10^{12} neutrons/sq. cm. sec. for 10 h and addition of the salt to 50 ml H_2O. A solution of 4.9 g KI in 20 ml H_2O was prepared and added with stirring to the above. The solution was diluted to 100 ml. Dilution of 5 ml of the solution to 100 ml gave 0.0025 M of Mayer reagent. The pH of a known volume of the alkaloid solution was made neutral or slightly acidic. Mayer reagent was added in small increments, and the mixture was stirred for 20-30 sec. and centrifuged. A known volume of the clear layer in the centrifugate was transferred into a spiral surrounding a cylindrical counter and the radioactivity was determined. The procedure was repeated several times to allow a plot of counts per min. vs. ml of Mayer reagent to be made. The equivalence point was sharply defined. Nitrate or sulfate ions do not interfere. Strychnine can also be determined in alcoholic solutions and in the presence of $\leqq$ 0.5 g brucine.

Strychnine and brucine, 5-30 γ each, are determined radiometrically in tincture of nux vomica (containing 0.224% total alkaloids) by measuring the β-activity of iodinated alkaloid - ^{131}I ppts, after separating strychnine and brucine by TLC on plates coated with Al_2O_3 without a binding agent (198). The method was carried out as follows:

Apply the solution containing strychnine and brucine, develop the chromatogram with Et_2O - EtOH (95:5), dry at 25°, spray the chromatogram with 1% iodine in $CHCl_3$, mark the spots, and remove excess iodine by heating the plate at 105°. Remove the Al_2O_3 containing the marked strychnine and brucine spots with a micropipet by applying gentle suction. Convert the alkaloids

to the respective acetate salts by warming the Al_2O_3 containing spot with 5 ml of 2% HOAC, filter the Al_2O_3 on ashless filter paper. Ppt strychnine and brucine separately with 2.5 ml of acidified aqueous ^{131}I solution, filter the ppts, measure the β-activity of the ppts, and find the concentration of both alkaloids from the respective calibration curve. The β-count vs. concn. curve is linear for 5-30 γ of strychnine and brucine.

A method for determination of strychnine in blood by double isotopic labeling was described (199). Double-labeled (^3H, ^{14}C) strychnine methiodide was prepared. For the assay, add strychnine stock solution and (or) water to 1 ml human citrated blood to make total volume of 2 ml, then add 0.2 ml (9600 counts/min) of a strychnine solution in absolute EtOH and 0.2 ml of absolute EtOH. Evaporate at 60° in vacuo nearly to dryness. Add 20 ml EtOH and repeat the evaporation. Add 1 ml of methyl iodide - ^3H solution (absolute EtOH) ($\sim$30 c./ml), seal the mixture in a glass tube, heat at 60° for 4 h, open the tube, and evaporate to 0.1 ml. This was paper chromatographed by a descending technique using EtOAC-MeOH-28% NH_4OH (100:10:15); unlabeled strychnine and double-labelled strychnine (for quenching corrections) were also chromatographed simultaneously with the sample. The amount of ^{14}C and ^3H in each spot was determined, using the percent recovery of ^{14}C to correct for losses during work up, the amount of strychnine - ^3H and thus the amount of strychnine in the original sample was calculated. It was reported that strychnine can be analyzed more accurately by this method than by more conventional procedures.

Another method for determination of *strychnos* alkaloids by radiometric titrations with potassium thallium iodide ($K^{204}TLI$)reagent was discussed (200). The sample solution containing 0.01 N acid was reacted with excess titrant within a total solution volume of 5 ml. The ppt of (Alkaloid H) TLI_4 was separated by centrifuging and the β activity of a supernatant aliquot was measured by using a Geiger counter. The sensitivities were $5x10^{-5}$ - $2x10^{-3}$ M for strychnine and brucine. The errors were within ±2.0% for determining 4-6 mg alkaloid alone and they were within ±3.2% for determining 7-15 mg alkaloid in pharmaceutical preparations.

7.8 Polarographic Determination

The catalytic polarographic waves of strychnine in buffers pH 8 were used for its determination in 10^{-4} - 10^{-5} M in pharmaceutical preparations, in solutions (1 ml) and tablets (after paper chromatographic separation from vitamins and sugars (201).

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VIDARABINE

Wen-Hai Hong, Tsun Chang and Robert E. Daly

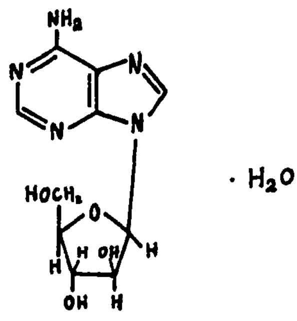
1. Introduction

1.1 History

Vidarabine is a purine nucleoside which does not occur naturally. It was first synthesized by Lee, et. al., as a potential anticancer agent in 1960 (1.2). Its antiviral activity against herpes simplex virus (HSV) and vaccinia virus (VV) in cell culture was first demonstrated by Privat de Garilhe and DeRudder in 1964 (3). Vidarabine has subsequently been obtained from fermentation cultures of Streptomyces antibioticus, tested and developed by Warner-Lambert/Parke-Davis Company (4). It has been used for the treatment of herpes simplex virus types 1 and 2, varicella-zoster, and vaccinia viruses infections (5).

1.2 Name, Formula, Molecular Weight

Vidarabine is a U.S. Adopted Name (USAN). Its chemical name is 9-(β-D-arabinofuranosyl)adenine monohydrate. It is also called adenine arabinoside, spongoadenosine, ara-A, vira-A and CI-673 (Parke-Davis).



$C_{10}H_{13}N_5O_4 \cdot H_2O$ MW: 285.27

1.3 Appearance, Color, Odor

Vidarabine is an odorless, white crystalline solid.

2. Physical Properties

2.1 Melting Range

Vidarabine melts in the range of 262° and 270°C.

2.2 Infrared Spectrum

The infrared spectrum of vidarabine presented in Figure 1 was taken as a 0.5% dispersion of vidarabine in potassium bromide using a Perkin-Elmer Model 621 infrared spectrophotometer. Table I gives the infrared assignments which are consistent with the structure of vidarabine.

TABLE I

INFRARED SPECTRAL ASSIGNMENTS FOR VIDARABINE

FREQUENCY (cm^{-1})	ASSIGNMENTS
3550 and 3450	NH stretch
3400 - 3200	OH stretch
1640	NH_2 bending
1140 - 1040	various C-O-C stretchings

2.3 Ultraviolet Spectrum

The ultraviolet spectrum of vidarabine in water is shown in Figure 2. Table II summarizes the ultraviolet spectral data for vidarabine (6).

TABLE II

ULTRAVIOLET SPECTRAL DATA FOR VIDARABINE

pH	λ MAX (nm)	ε
1.0	257.5	12,700
7.0	259.0	13,400
13.0	259.0	14,000

2.4 Nuclear Magnetic Resonance Spectrum

The NMR spectrum of vidarabine is shown in Figure 3. The spectrum was obtained with a Varian Model 60 MC spectrometer. Deuterated dimethylsulfoxide was used as the solvent with tetramethylsilane as an internal standard. The assignments, chemical shifts and multiplication are described in Table III and are consistent with the structure of vidarabine.

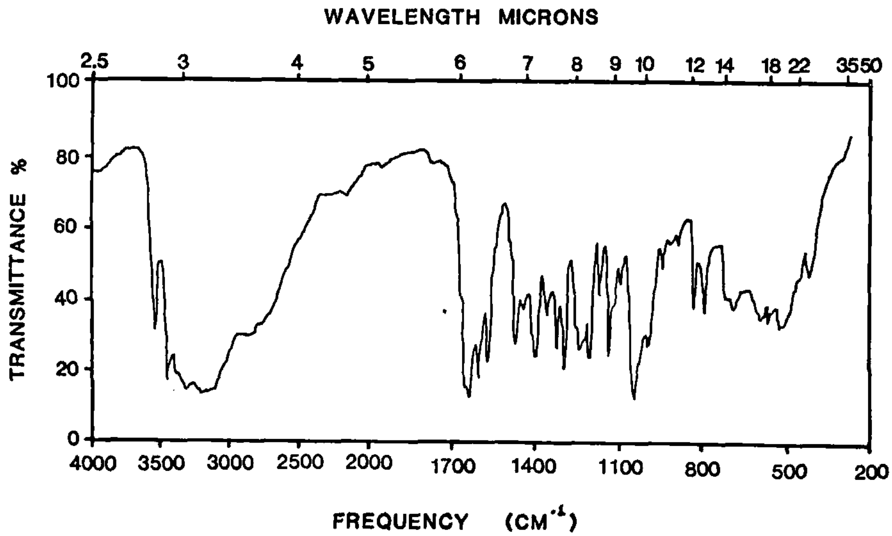


Figure 1 Infrared Spectrum of Vidarabine

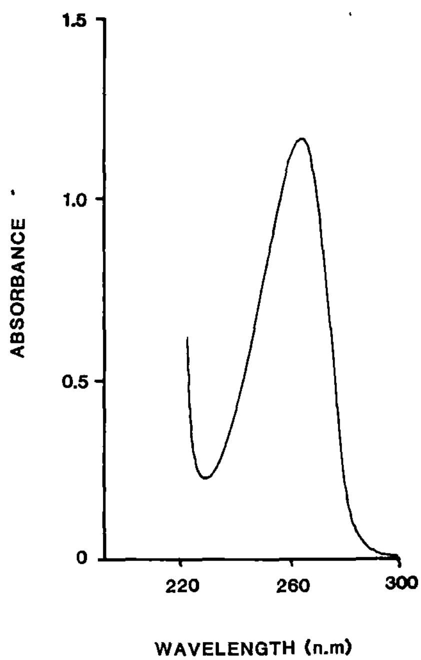


Figure 2 Ultraviolet Spectrum of Vidarabine in Water

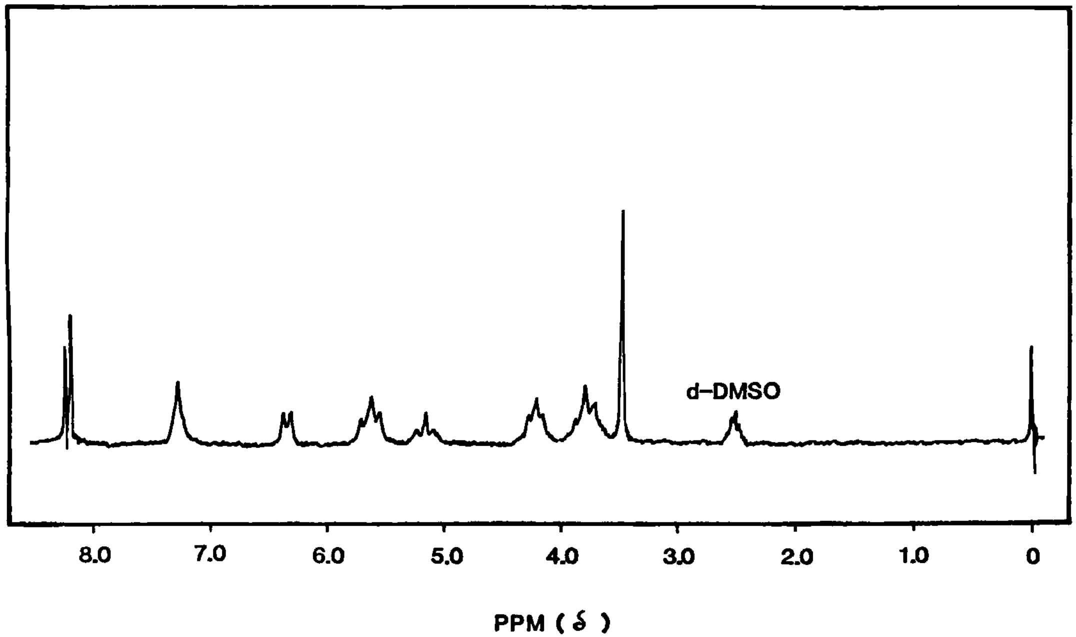


Figure 3 Nuclear Magnetic Resounance Spectrum of Vidarabine

TABLE III

NUCLEAR MAGNETIC RESONANCE ASSIGNMENTS FOR VIDARABINE

CHEMICAL SHIFTS ppm	MULTIPLICITY	NO. OF PROTONS	ASSIGNMENTS
8.27, 8.21	Doublet	2	Two aromatic protons, one at C-2, and one at C-8 of adenine
7.29	Singlet	2	Two protons of the 6-amino group
6.38, 6.31	Doublet	1	Anomeric proton at C-1 split by the cis proton at C-2 with a J of 4.2 cps
5.63	Triplet	2	A superposition of two doublets due to the hydroxyl protons of C'-2 and C'-3
5.18	Triplet	1	C'-5-hydroxyl proton being split by the C'-5 protons
4.22	Multiplet	2	Two of the five protons at C'-2, C'-3, C'-4 and C'-5 being split by the neighboring protons
3.8, 3.71	Multiplet	3	Three of the five protons at C'-2, C'-3, C'-4 and C'-5

2.5 Optical Rotation

The specific rotations of a 1% (w/v) vidarabine solution in dimethylformamide are presented in Table IV.

TABLE IV

SPECIFIC ROTATIONS OF A 1% (w/v) VIDARABINE SOLUTION IN DIMETHYLFORMAMIDE

WAVELENGTH (nm)	$[\alpha]^{25}$
589 (Na_D)	-2.5°
578	-2.9°
546	-12.5°
436	-30.4°
365	-76.2°

Vidarabine Lot RxX41064 was used

The specific rotations of a 1% (w/v) solution of vidarabine and its optical enantiomorph, 9-(β-L-arabinofuranosyl) adenine, in dimethylsulfoxide are presented in Table V. As shown, the rotations for the enantiomorph are opposite in sign. The enantiomorph was prepared according to the procedure given by Glaudemans and Fletcher (7).

TABLE V

SPECIFIC ROTATIONS OF A 1% (w/v) VIDARABINE AND ITS OPTICAL ENANTIOMORPH 9-(β-L-ARABINOFURANOSYL) ADENINE, SOLUTIONS IN DMSO

	$[\alpha]^{25}$	
WAVELENGTH (nm)	VIDARABINE	OPTICAL ENANTIOMORPH
589 (Na_D)	-9.7°	+9.2°
578	-10.5°	+9.8°
546	-12.5°	+12.0°
436	-30.4°	+30.2°
365	-76.2°	+76.4°

2.6 Solubility

Since vidarabine behaves as both a weak acid and a weak base, its solubility in water depends on pH as well as other factors such as temperature and state of hydration. The aqueous solubility of vidarabine, expressed in terms of the hydrate, at various pHs is shown in Figure 4. As seen, vidarabine behaves as a neutral compound over the pH region 5 to 10.5. Over this pH region the solubility is invarient and equal to 0.44 mg per ml (23°C). Also, as seen, rather acidic or basic pHs are required to significantly increase its solubility.

Hydration influences vidarabine solubility as shown in Figure 5. The solubility of the anhydrate has not been accurately determined since conversion of anhydrate to hydrate occurs very rapidly as shown in Figure 5. However, the anhydrate is at least 5 times more soluble than the hydrate in water.

Solubilities of vidarabine at various temperatures are given in Figure 6. Since the van't Hoff relationship is applicable, these data indicate the monohydrate is the stable solid phase in equilibrium with solution at temperatures in the range of 25° to 100°C.

In regard to nonaqueous solvents, the solubility of vidarabine in such commonly used organic solvents as alcohols, esters, chlorinated hydrocarbons, etc., is very low. Polar solvents such as dimethylsulfoxide, and dimethylformamide are good solvents for vidarabine as is indicated by the data given in Table VI.

TABLE VI

SOLUBILITY OF VIDARABINE IN DIMETHYLFORMAMIDE, DIMETHYLSULFOXIDE AND DIMETHYLSULFOXIDE - WATER MIXTURE AT 25°C

SOLVENT	SOLUBILITY (mg/ml)
Dimethylformamide	20
Dimethylsulfoxide	250
DMSO/Water (v/v)	
90/10	150
75/25	20.5
50/50	3.4
25/75	1.2

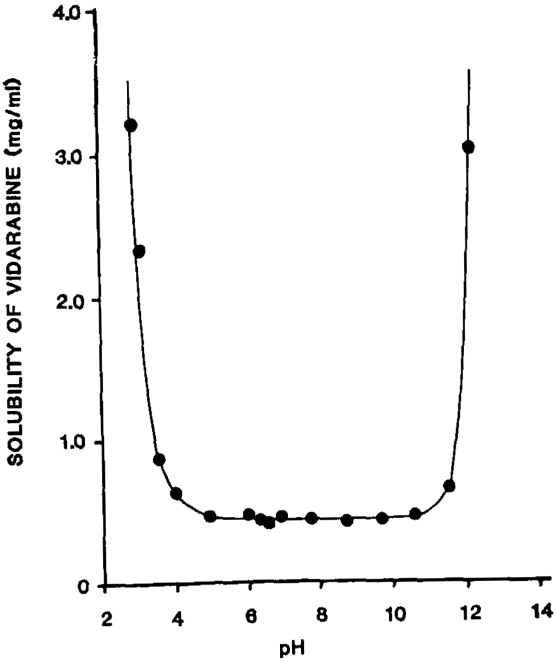


Figure 4 Solubility Profile of Vidarabine

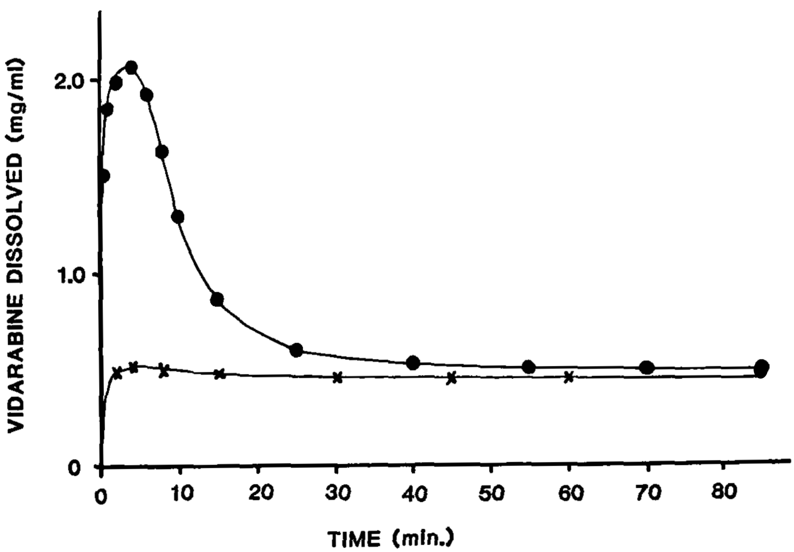


Figure 5 Dissolution of Vidarabine in Water at Room Temperature

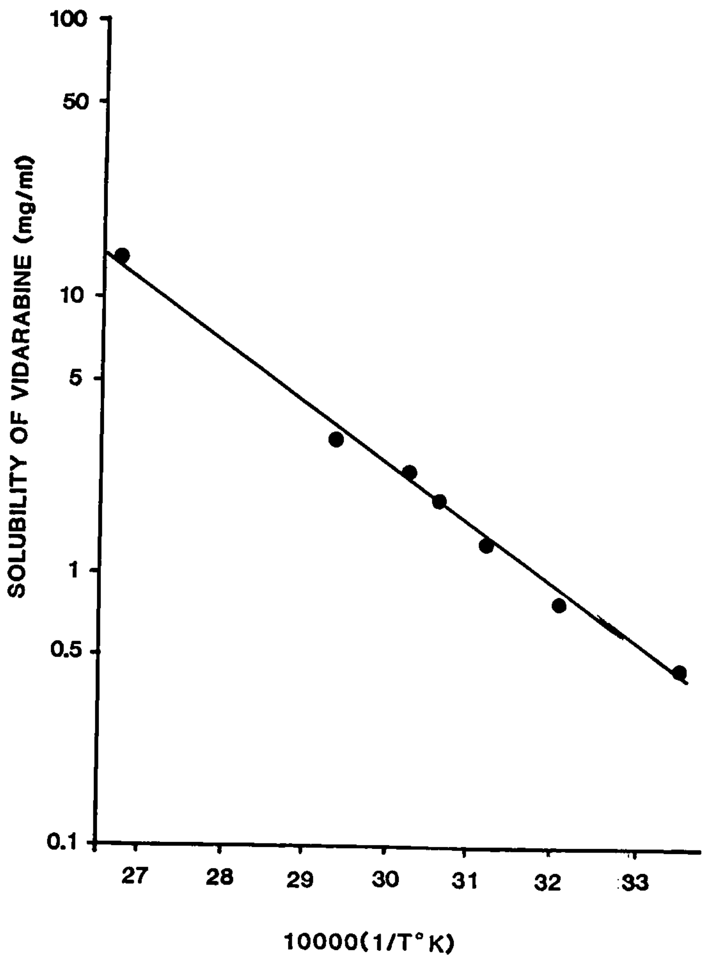
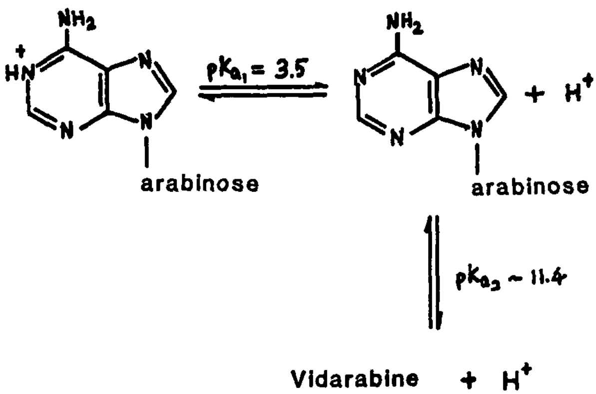


Figure 6 A van't Hoff Plot of Vidarabine Solubility Data

2.7 pKa Value

Vidarabine is an amphoteric compound which can act as both a weak acid and a weak base. The protonated form of vidarabine has a pKa of 3.5. This value is similar to that reported for adenosine, (3.6) as would be expected (8 - 12). The protonation site for vidarabine has not been established but anology with adenosine suggests the nitrogen in position 1 is involved (13).



Vidarabine's second pKa (11.4) also approximates that reported for adenosine (12.34). This ionization is likely associated with a hydroxyl group (position unknown) contained in arabinofuranosyl moiety.

2.8 Thermal Analysis (DSC and TGA)

A differential scanning calorimetry curve was obtained (Figure 7) on a Perkin-Elmer DSC-2C differential calorimeter. Using nitrogen as the purge gas, the scan was performed at a rate of 10°C/minute from 50° to 300°C. The DSC curve reveals a broad endothermic dehydration peak (onset 123.1°C) and a single sharp endothermic melting peak (onset 263.9°C) followed by decomposition of the sample.

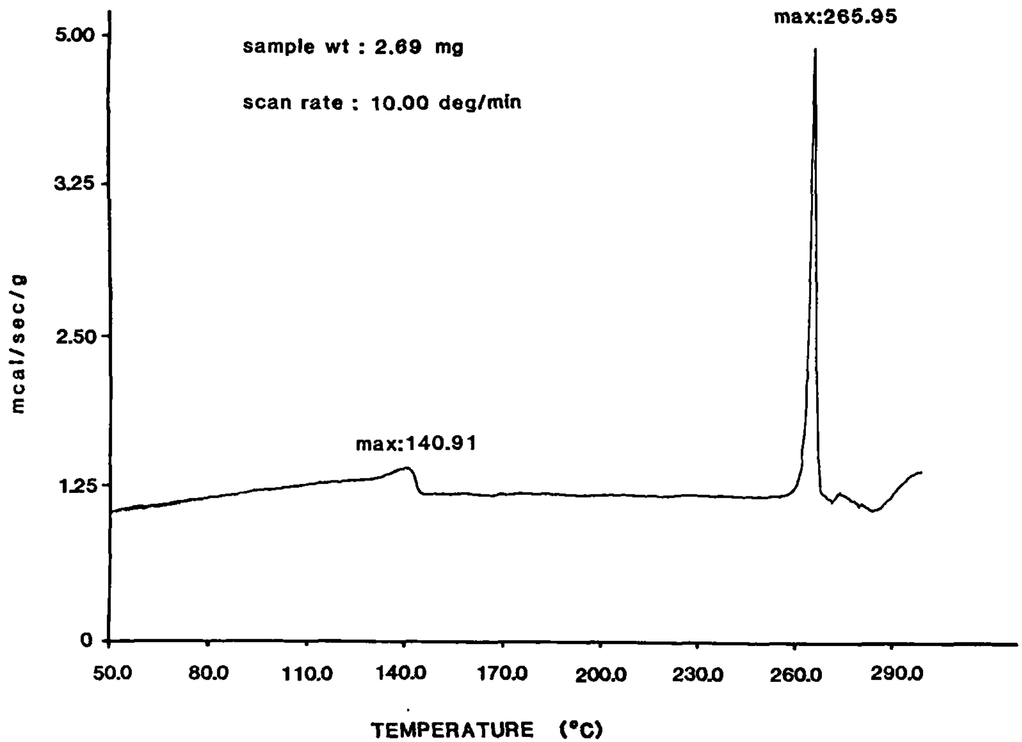


Figure 7 Differential Scanning Calorimetry Curve of Vidarabine

A thermogravimetric analysis curve was obtained using a Perkin-Elmer TGS-2 thermogravimetric analyzer. The analysis was performed at a rate of 10°C/minute from 35.8° to 230°C. The thermogravimetric analysis curve (Figure 8) shows a weight loss of 6.32% from 30° to 110°C, which conforms the presence of one mole of water.

3. Synthesis and Production

Vidarabine has been synthesized by two different methods. The first involves a cleavage reaction of 9-(2,3-anhydro-β-D-lyxofuranosyl)adenine, which is derived from 9-β-D-xylofuranosyladenine (1). The second is a condensation of benzoyladenine with 2,3,5-tri-O-benzyl-β-D-arabinosyl-chloride (7). Both methods involve laborious condensation of adenine with a sugar derivative. Ikehara et.al (14) synthesized vidarabine from naturally occurring adenosine employing a selective cleavage reaction of 8,2'-anhydroadenosine by hydrogen sulfide, and desulfurization of the resulting 6-amino-9-β-D-arabinofuranosylpurine-8-thiol.

Production of vidarabine by fermentation was conducted until the late 1960's by Parke Davis and Company using a strain of Streptomyces Antibioticus (4). The harvested beer was filtered, concentrated under reduced pressure and cooled at 2 - 10°C to effect optimal precipitation of vidarabine. The deposited crystals were filtered off and recrystallized from boiling water. The thrice crystallized vidarabine was filtered off, washed with cold water, and cold acetone, and then dried in vacuo at room temperature.

4. Impurity

The impurity most likely in vidarabine is the deaminated analog, 9-(β-D-arabinofuranosyl) hypoxanthine. This impurity can be detected by TLC using silica gel plate F-254 with the solvent system ethylacetate-isopropyl alcohol-water (195:70:30) or chloroform-methanol (100:30). The R_f values will vary depending on temperature, TLC plates, etc., but the separation of the two components is consistent. The chloroform-methanol system provides the most reproducible results.

SOLVENT SYSTEM	R_f VALUE VIDARABINE	DEAMINATED ANALOG
Ethylacetate-isopropyl alcohol-water	0.42 - 0.58	0.33 - 0.49
Chloroform-methanol	0.38 - 0.39	0.14 - 0.17

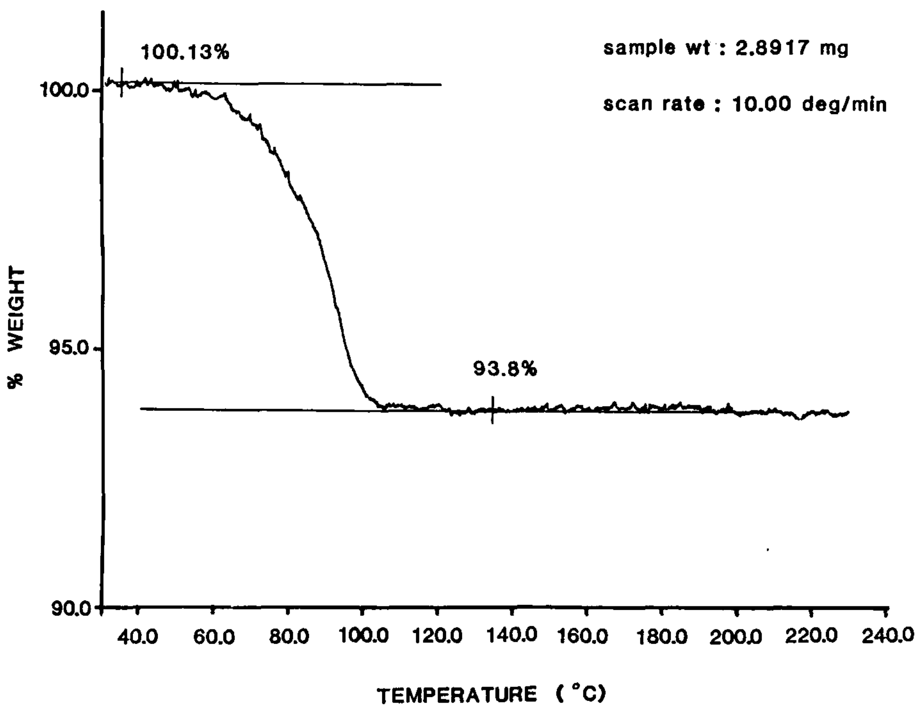


Figure 8 Thermogravimetric Analysis Curve of Vidarabine

Inspection of the developed plates under short wavelength UV light reveals blue spots for either vidarabine or the deaminated analog; a minimum of 0.25 μg of either compound per spot is necessary to detect the purine derivatives. Elution of each spot or zone from the TLC plate with aqueous methanol (50%) and examination of each eluate in the UV spectrophotometer gave the respective UV spectra for vidarabine (λ max at 260 nm) or the deaminated analog (λ max at 249 nm). A minimum of 6 μg of material is required to obtain the spectrum.

5. Stability

The solution stability of vidarabine in 0.1 **N** hydrochloric acid at 100°C was followed by spectrophotometric and polarimetric methods (15). Both methods gave the same results indicating the hydrolysis only involved the cleavage of the **N**-glycoside linkage without racemization or other side reactions. This result was also confirmed by TLC analysis.

6. Metabolism and Disposition

The disposition of vidarabine has been studied in laboratory animals and in man following parenteral administraiton of tritium labeled drug.

Vidarabine is rapidly and extensively deaminated to the less active metabolite, ara-hypoxanthine (ara-Hx) (16-18) followed by subsequent oxidation of the purine ring (19) (Figure 9). A small fraction of vidarabine is phosphorylated by cellular enzymes to yield ara-ATP (20). Ara-Hx represents the major metabolite in plasma, urine, erythrocytes, and tissues after the administration of tritium labeled drug.

7. Pharmacokinetics

Animal Studies

Following intravenous administration of tritium labeled vidarabine to dogs and monkeys, plasma nonvolatile tritium ($NV\text{-}H^3$) declined rapidly with estimated half life of 30 minutes and 2 to 4 hours, respectively. Loss of the tritium label resulted from further oxidation of ara-Hx which was evident from the presence of tritiated water in plasma and urine. Application of a specific HPLC assay revealed that greater than 90% of the plasma $NV\text{-}H^3$ could be accounted for by ara-Hx. Only traces of vidarabine was detected 5 minutes after iv injection. Existing evidence also indicate that vidarabine or metabolites equilibrated rapidly between plasma and erythrocytes followed by slow incorporation of drug

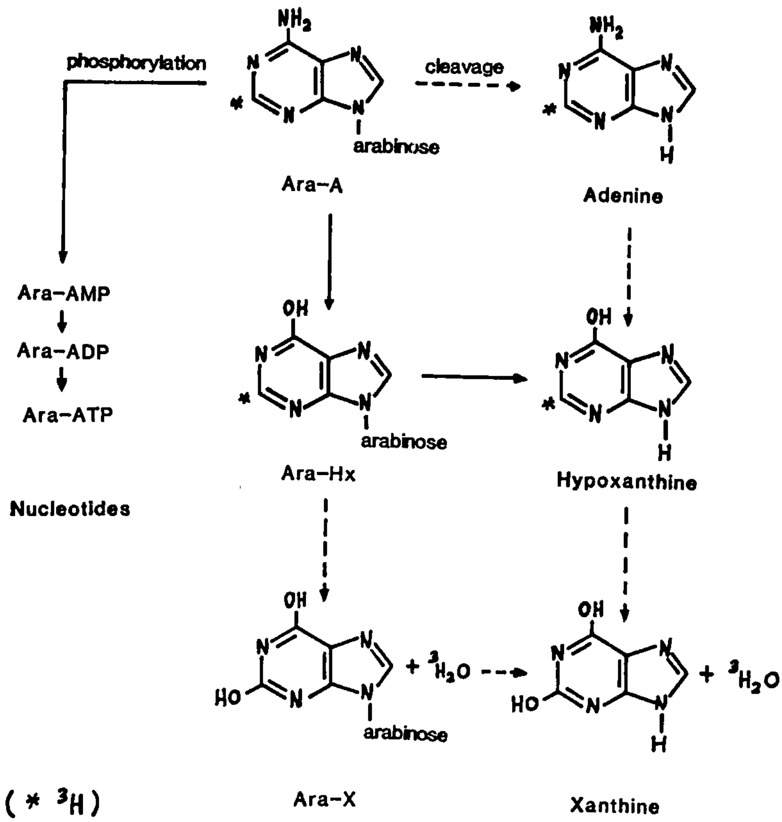


Figure 9 Pathways of Metabolic Disposition of Vidarabine

related tritium label into the erythrocytes. Urinary excretion accounted for 20% and 90% of the administered dose in dogs and monkeys, respectively, with less than 1% of the dose recovered in feces. Chronic once daily iv doses failed to show any evidence of drug (or metabolite) accumulation (17).

Absorption following intramuscular administration of vidarabine suspension was slow and prolonged resulting in lower plasma levels in both animal species. Half life values of 12 to 24 hours were reported (17).

Vidarabine was extensively distributed to tissues. Following parenteral administration of ^3H-ara-A, highest concentration of tritium were found in the kidneys with progressively lower concentrations in the spleen, liver, heart, skeletal muscle, lung, brain, and blood plasma (17).

Clinical Studies

Glazko **et al** (17) reported that following iv administration of tritium labeled vidarabine, the plasma NV-^3H half life ranged from 3 to 5 hours. Among infants and children receiving unlabeled iv doses of vidarabine, the maximal plasma level was on the order of 1-2 μg/ml and the half life was about 1.5-2.5 hr, shorter than in the adult. In adults receiving im vidarabine, the maximal plasma level was about 0.2-0.3 μg/ml and the plasma half-life about 10-16 hr.

With iv administration of vidarabine to humans, the maximum urinary excretion rate of ^3H occurred in the first 4 hr. after dosing with mean recovery of about 45 percent within 24 hr. Again as in animals, maxima were delayed after intramuscular administration.

LePage **et al** (21) studied the excretion of ^3H-labeled vidarabine and ara-Hx in human patients receiving rapid intravenous infusions (1/2-2 hr), continuous intravenous drips (24 hr), or intramuscular injections. They reported that in the two patients receiving rapid infusions, 90 percent of the drug was recovered in the urine in 24 hr. In the two patients receiving im injections, about 5 percent was recovered in 24 hr.

Kinkel and Buchanan (22) studied the plasma, cerebrospinal fluid, erythrocyte, and urine levels of vidarabine and ara-Hx following iv infusion in seven patients. Vidarabine was administered by either iv drip or by a constant rate infusion pump over a range of 4-10 days.

The levels of vidarabine appearing in the plasma were just barely above the limits of the HPLC assay. The levels of ara-Hx, however, rose promptly during the infusion period and then declined promptly when infusion was stopped. The elimination half lives in the patients observed were between 3 1/2 and 4 hr. The levels found on day 1 and day 5 were similar, indicating no accumulation or change in metabolism, distribution, or excretion over that period.

8. Methods of Analysis

8.1 Elemental Analysis

A typical elemental analysis of a sample of vidarabine lot RxX41064 is presented in Table VII.

TABLE VII

ELEMENTAL ANALYSIS FOR VIDARABINE

	% AS MONOHYDRATE		% AS ANHYDRATE	
ELEMENT	**THEORY**	**FOUND**	**THEORY**	**FOUND**
C	42.10	42.76	44.94	45.10
H	4.60	4.64	4.90	4.89
N	24.55	25.09	26.21	26.46
H_2O	6.32	5.19	0.00	0.00

8.2 Identification

The identity of vidarabine is established by infrared spectroscopy. The infrared absorption spectrum of a 0.5% dispersion in potassium bromide exhibits maxima only at the same wavelengths as that of a similar preparation of USP Vidarabine Reference Standard.

Ultraviolet spectroscopy is also used to identify vidarabine. The spectrum of a sample dissolved in water (approximately 15 μg per ml) scanned from 320 - 220 nm compares qualitatively to that of a vidarabine standard similarly prepared.

8.3 Loss on Drying

Dry about 100 mg of vidarabine in vacuum at 100°C and at a pressure not exceeding 5 mm of mercury for 4 hours; it loses between 5.0% and 7.0% of its weight (23).

8.4 Specific Rotation

Using a solution containing 10 mg of anhydrous vidarabine per ml in dimethylformamide and a polarimeter tube 1.0 decimeter in length, determine the specific rotation at 25°C at 365 nm. The value lies between -56.0° and 65.0° (23).

8.5 Ultraviolet Spectrophotometric Analysis

Vidarabine exhibits a UV absorption band with a maximum near 260 nm in water. Quantitation of the vidarabine content is based on the net absorbances at max of the assay preparation as compared to that of the standard preparation. The method is not stability indicating since adenine, a hydrolytic product, possesses a similar UV spectrum. However, separation of adenine from vidarabine prior to UV analysis can be achieved on an anionic ion exchanger using 0.1 M pH 10 borate buffer as the eluent (15).

8.6 Thin-Layer Chromatography

A number of thin-layer chromatographic systems have been reported for the identification of vidarabine and for the separation from its mostly likely impurities, adenine and 9- β-D-arabinofuranosylhypoxanthine (24,25). The systems are described below and their respective R_f values are tabulated.

System I

Plate: Silica Gel GF 254 plate, 250 μm

Mobile phase: isopropyl alcohol/ammonia/water (65: 10:25)

Detection: shortwave UV

System II

Plate: Silica Gel GF 254 plate, 250 μm

Mobile phase: lower phase of chloroform/methanol/ 3% acetic acid (3:2:1)

Detection: shortwave UV

8.6 Thin-Layer Chromatography (continued)

System III

Plate: Silica Gel GF 254 plate, 250 μm

Mobile phase: Lower phase of chloroform/methanol/15% ammonia (3:2:1)

Detection: shortwave UV

System IV

Plate: Plastic sheets (20 x 20 cm) coated with 10 μm of PEI-impregnated MN300 cellulose with fluorescent indicator

Mobile phase: 0.1 **M** boric acid

Detection: shortwave UV

TABLE VIII

THIN-LAYER CHROMATOGRAPHIC SEPARATION OF VIDARABINE, 9-β-D-ARABINOFURANOSYLHYPOXANTHINE AND ADENINE

	Rf VALUE		
SYSTEM	**VIDARABINE**	**9-β-D-ARABINOFURANOSYL HYPOXANTHINE**	**ADENINE**
I	0.67	0.60	0.74
II	0.26	0.16	0.43
III	0.44	0.18	0.60
IV	0.57	0.49	0.40

8.7 High-Pressure Liquid Chromatography (HPLC)

The following systems have been reported for the analysis of vidarabine in dosage formulations and biological fluids.

System I (23,26)

Column: 30 cm x 4 mm i.d., C_{18} column 5 or 10 μm

Mobile phase: Docusate sodium/glacial acetic acid/methanol (2.2:10:500), q.s. to 1000 with water.

8.7 High-Pressure Liquid Chromatography (HPLC) (cont.)

System I (23,26) (continued)

Temperature: ambient

Detection: 254 nm

Flow rate: 1.5 ml/ml

System II (17)

Column: 60 cm x 1.8 mm i.d., Aminex A-28 column

Mobile phase: 0.2 **M** pH 7.4 acetate buffer

Temperature: 65°C

Detection: 254 nm

Flow rate: 15 - 20 ml/hr.

System III (27)

Column: 15 cm x 3.7 mm i.d., Aminex A-28 column

Mobile phase: 0.01 **M** pH 6.3 borate buffer

Temperature: 60°C

Detection: 254 nm

Flow rate: 0.5 ml/min.

System IV (28)

Column: 15 cm x 4.6 mm i.d., Ultrasphere ODS 5 µm column

Mobile phase: 0.01 **M** potassium dihydrogen phosphate (Solution A) and 30% methanol in 0.01 **M** potassium dihydrogen phosphate (Solution B). Linear gradient of 10 - 30% of Solution B in Solution A, achieved in 15 minutes.

Temperature: Ambient

Detection: 254 nm

Flow rate: 1.0 ml/min.

8.7 High-Pressure Liquid Chromatography (HPLC): (cont.)

System V (29)

Column: 25 cm x 4.6 mm i.d., Li-Chrosorb RP-8 column

Mobile phase: 20 ml of acetonitrile in 480 ml of 0.005 **M** sodium pentanesulfonate buffer, pH 7.2

Temperature: 40°C

Detection: 250 nm

Flow rate: 1.0 ml/min.

System VI (30)

Column: 25 cm x 4.6 mm i.d., Whatman Partisil 10/ODS-3 column, preceded by a Brownlee C-18 guard catridge system

Mobile phase: 0.01 **M** potassium dihydrogen phosphate, pH adjusted to 3.25 ± 0.005 with acetic acid/acetonitrile (95:5)

Temperature: Ambient

Detection: 254 nm

Flow rate: 1.0 ml/min.

8.8 Microbiological Assay

Vidarabine is assayed microbiologically with Pichia membranae-faciens as the test organism using the agar diffusion method. The potency of a sample of vidarabine is then obtained by comparing the zone of inhibition with those of the standard preparation (31). The sensitivity of this method is approximately 20 µg per ml of the test solution.

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ZOMEPIRAC SODIUM

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Franjo Kajfež, Zlatko Meić

1. Foreword, History, Therapeutic Category

Zomepirac is a benzoylpyrrole acetic acid derivative which is chemically closely related to the non-steroidal anti-inflammatory agent tolmetin /1/. It is a potent analgesic with a predominantly peripheral action mediated through prostaglandin synthetase inhibition. Zomepirac sodium has been shown to be effective in many chronic and acute pain conditions; it is at least as effective as usual therapeutic doses of aspirin, codein alone or with aspirin, phenacetin and caffein, dextropropoxyphene with paracetamol or orally administered pentazocine. Additionally, zomepirac sodium may provide analgesia comparable to that of standard doses of intramuscular morphine. However, serious side effects have been found during the therapy with zomepirac sodium which resulted in withdrawal pf this drug by the manufacturer (McNeil Lab. USA) in 1983. It is expected that zomepirac sodium will be again available but only for those patients where treatment with other non--steroidal anti-inflammatory agents is not successfull.

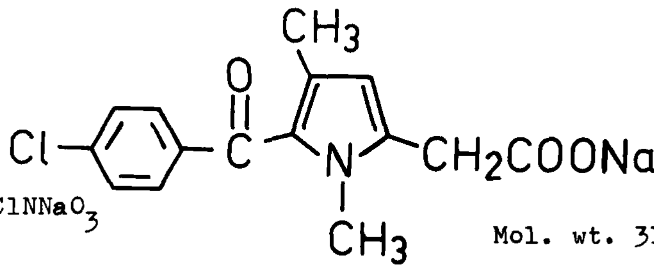
2. Description

2.1. Name, Formula, Molecular Weight

Chemical Name: sodium salt of 1,4-dimethyl-5-(p-chlorobenzoyl)-1H-pyrrole-2-acetic acid.

Generic name: Zomepirac sodium

Trade name: Zomax



$C_{15}H_{13}ClNNaO_3$

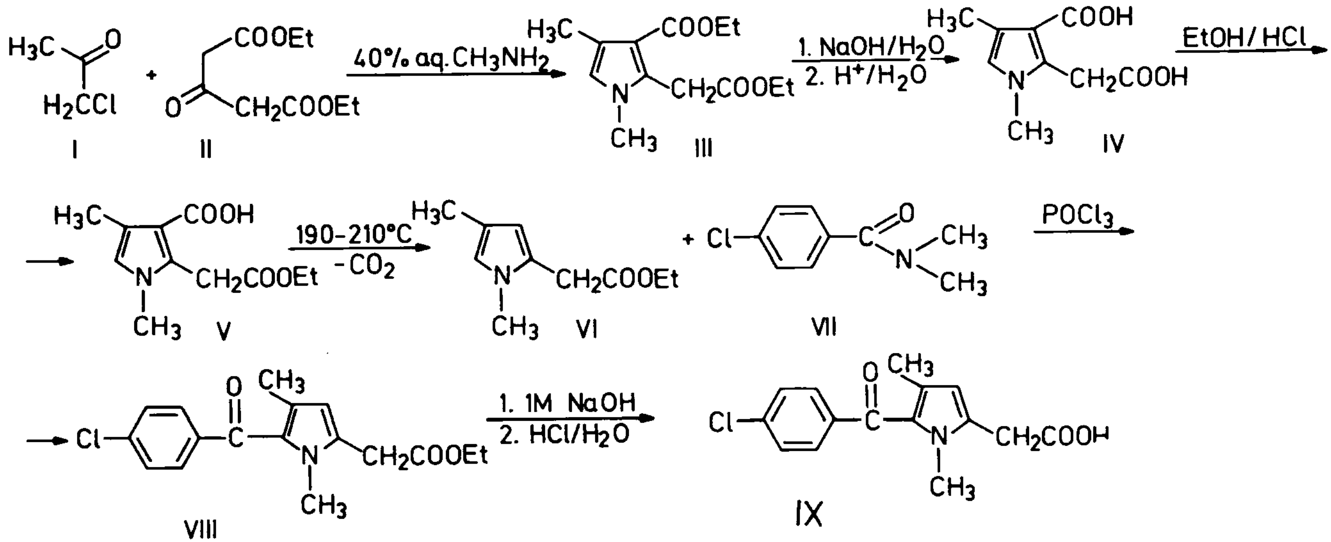
Mol. wt. 313.56

2.2. Appearance, Color, Odor

Zomepirac is marketed as the sodium salt dihydrate; pale yellow crystalline, odorless powder. Free acid appears as white, odorless crystalline powder.

3. Synthesis

The first description of a synthesis of zomepirac (see Scheme I) was published by Carson and Wong /1,2/ in 1973. The first step in this procedure is a modified Hantzsch pyrrole synthesis from chloroacetone (I), diethyl 3-oxoglutarate II and 40% aqueous methylamine which gave the diester III. Saponification of this product gave



H3C
O
H2CCl
I
+
COOEt
O
CH2COOEt
II
40% aq.CH3NH2
H3C
COOEt
N
CH2COOEt
CH3
III
1. NaOH/H2O
2. H+/H2O
H3C
COOH
N
CH2COOH
CH3
IV
EtOH/HCl
H3C
COOH
N
CH2COOEt
CH3
V
190-210°C
-CO2
H3C
N
CH2COOEt
CH3
VI
+ Cl
C
O
N
CH3
CH3
VII
POCl3
Cl
O
C
CH3
N
CH2COOEt
CH3
VIII
1. 1M NaOH
2. HCl/H2O
Cl
O
C
CH3
N
CH2COOH
CH3
IX

SCHEME 1.

the diacid IV which was selectively esterified to give the monoester V. The latter was decarboxylated by heating at 190-200°C. The resulting pyrrole derivative VI was aroylated with N,N-dimethyl-p-chlorobenzamide (VII) and phosphoryl chloride (Vilsmeier aroylation) to give compound VIII, which was finally saponified to zomepirac. The overall yield is about 6%.

Recently, two modifications of this synthetic route appeared in the literature describing selective one pot decarboxylation /3/ and decarbethoxylation /4/ of intermedate III to VI.

The syntheses of deuterium /5/ and tritium /6/ labelled zomepirac were also published.

4. Physical Properties

4.1. Spectra

4.1.1. Infrared

The IR absorption spectra of zomepirac and its sodium salt dihydrate are shown on Fig. 1. Spectra were recorded from KBr-pelleted samples on the Pye-Unicam SP-3 infrared spectrophotometer.

On the spectrum of zomepirac (A), the following characteristic bands should be noted (cm^{-1}): 3100 (C-H stretching, phenyl and pyrrole rings), 2500-3000 (carboxylic O-H stretching), 1720 and 1700 (ketone and carboxylic C=O stretching respectively). Further bands at 1604 and 1475 cm^{-1} can be attributed to the pyrrole ring skeleton vibrations, and the strong bands at 760 and 750 cm^{-1} to the pyrrole C-H out-of-plane vibrations.

On the spectrum of zomepirac sodium salt dihydrate (B), the O-H stretching vibration band appears at 3500 cm^{-1}, due to the presence of crystal water. Strong bands at 1580 and 1370 cm^{-1} are caused by symmetric and asymmetric stretching vibrations of the carboxylate anion.

4.1.2. Ultraviolet

Fig. 2 shows the UV spectra of zomepirac measured in methanol, 0.1 M hydrochloric acid and 0.1 M sodium hydroxide solution. Slight shift of the longer wavelength maxima toward higher values should be noted by the change of the medium from acidic (321 nm) over neutral (322 nm) to basic (328 nm). Shorter wavelength maxima at 252 nm is also shifted to 258 nm in basic solution and 261 nm in hydrochloric acid.

4.1.3. Mass

The mass spectra of zomepirac Fig.(3A) and its sodium salt dihydrate (Fig. 3B) were recorded on a Kratos

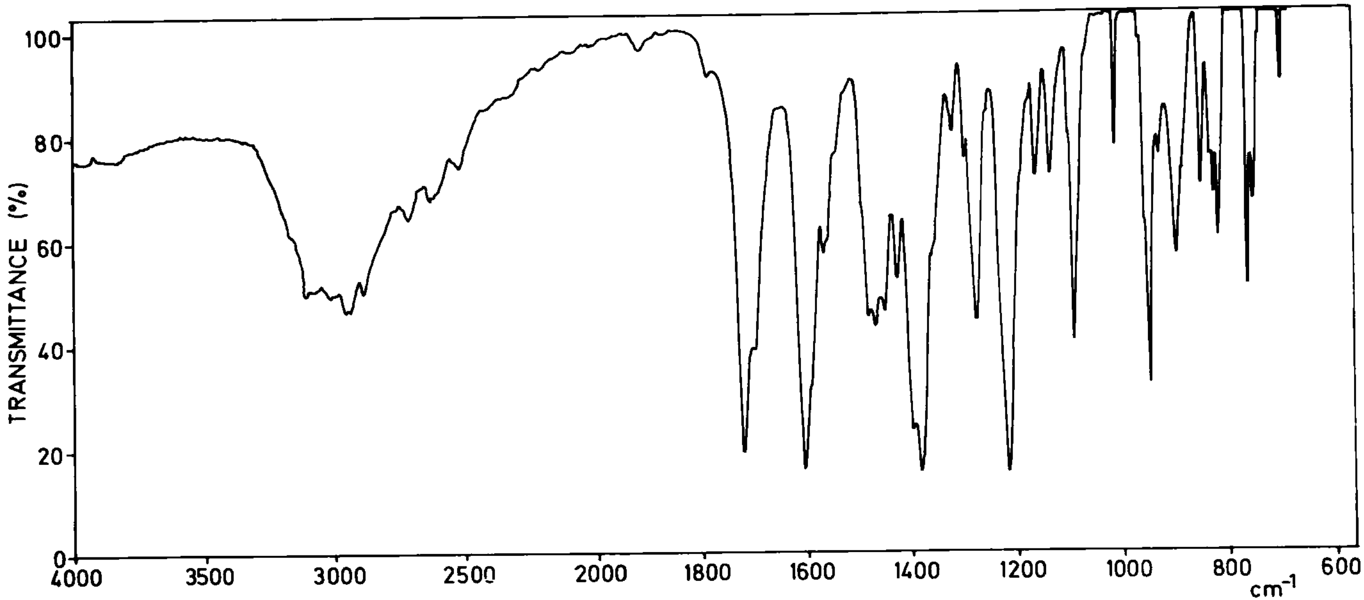


Fig. 1. A- Infrared spectrum of zomepirac in KBr pellet.
Instrument: Pye-Unicam SP 3-2oo spectrophotometer.

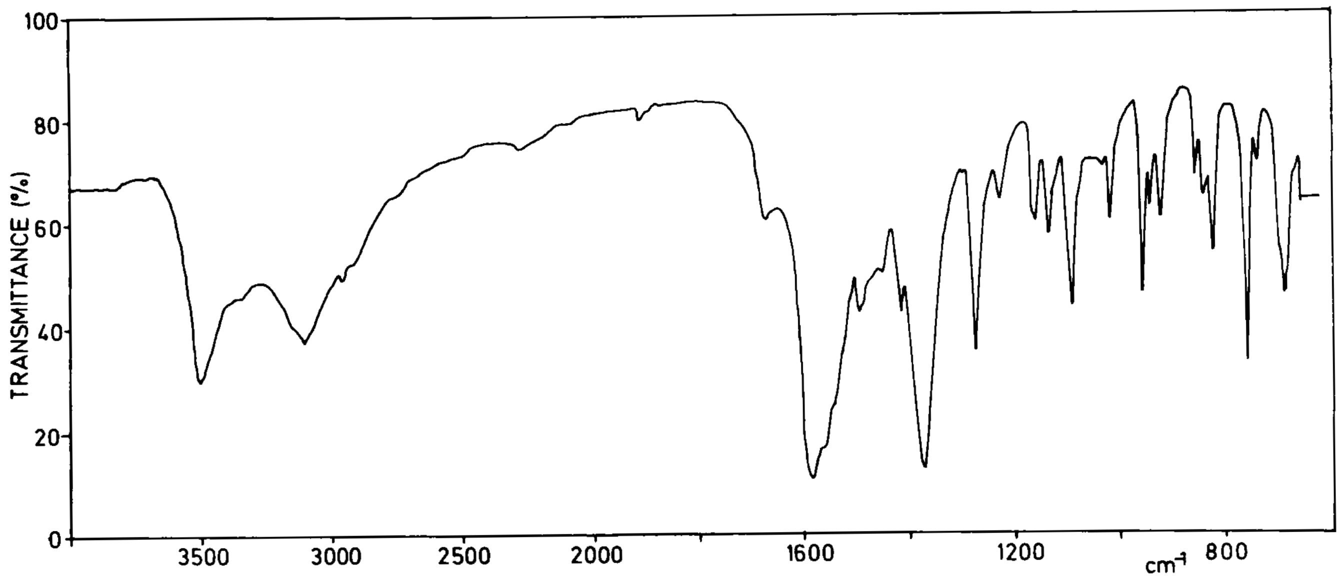


Fig. 1. B- Infrared spectrum of zomepirac sodium salt dihydrate in KBr pellet.
Instrument: Pye-Unicam SP3-2oo spectrophotometer.

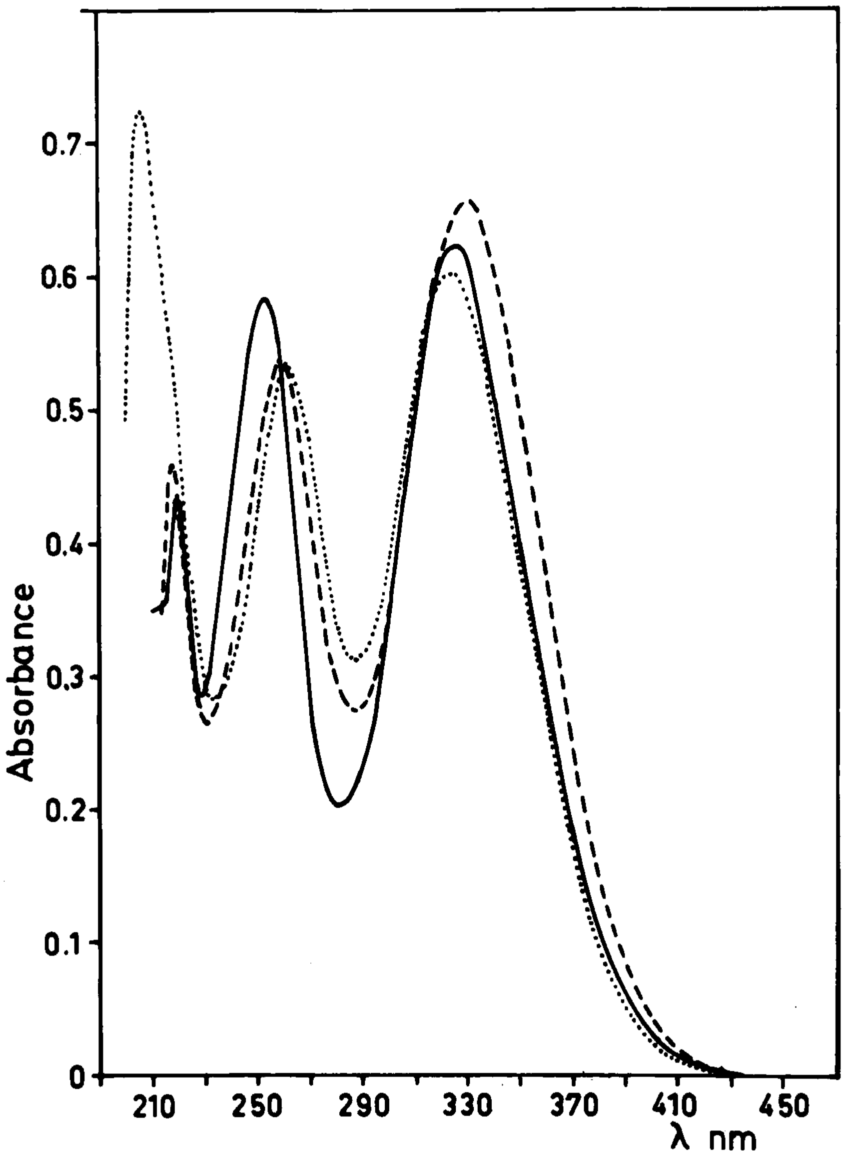


Fig. 2. Ultraviolet spectra of zomepirac in:
——— o.1 M sodium hydroxide,
...... o.1 M hydrochloric acid, and
------ methanol solution.

Instrument: Pye-Unicam SP8-1oo.

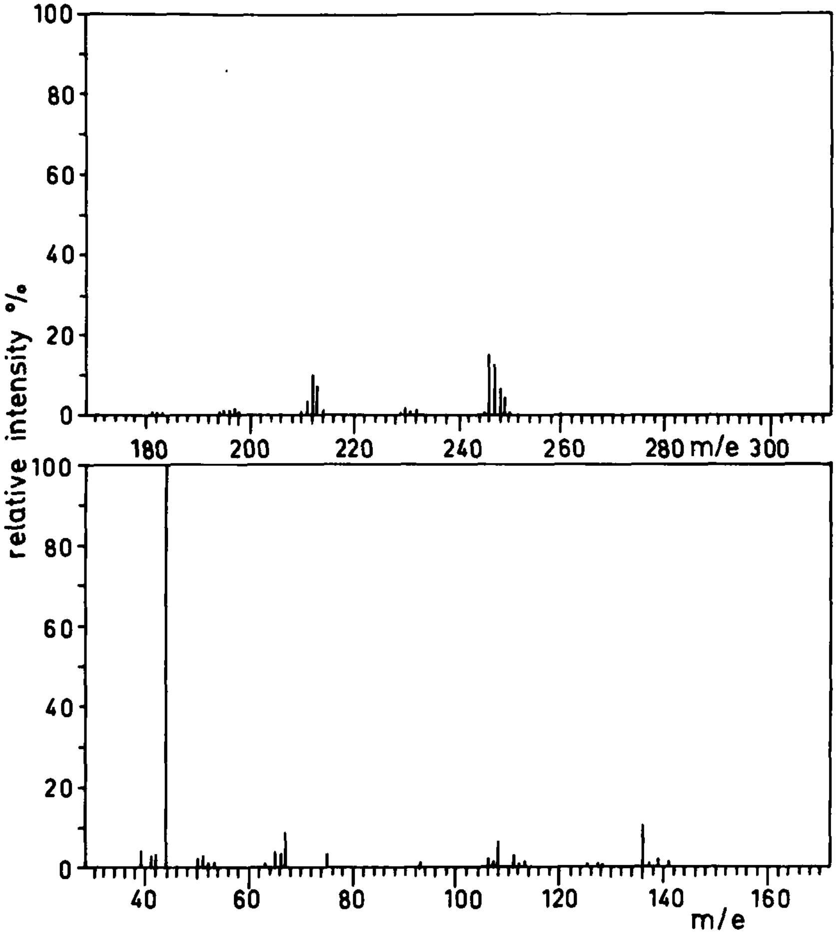


Fig. 3. A- Mass spectrum of zomepirac.
Instrument: Kratos MS-25.

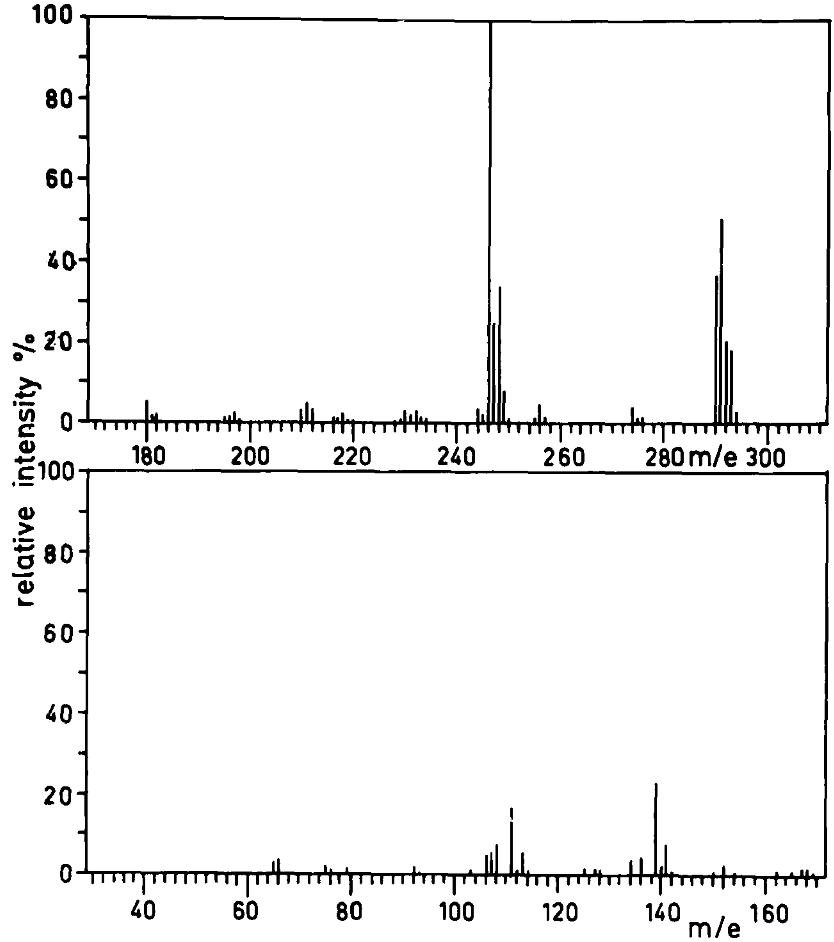


Fig. 3. B- Mass spectrum of zomepirac sodium salt dihydrate. Instrument: Kratos MS-25.

MS-25 spectrometer linked to DS 5oS data system using direct insertion probe.

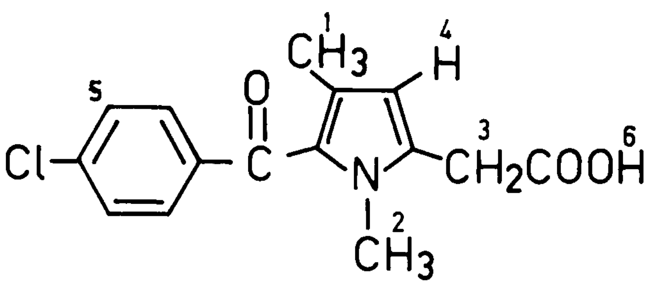
In the mass spectrum of zomepirac, the molecular ion appears at m/e 291 with 51% of relative intensity. The base peak at m/e 246 is apparently formed by decarboxylation. Other prominent peaks are m/e 139 (C_7H_4OCl) and m/e 111 (C_6H_4Cl) formed by fragmentation of the carbonyl group. Small peaks at m/e 276 and m/e 256 resulted from N--demethylation and the cleavage of chlorine atom in the p-position of the phenyl ring respectively.

The main characteristics of the mass spectrum shown on Fig. 3B is the absence of molecular ion. The base peak at m/e 247 results from the decarboxylation process. Further fragmentation can be interpreted as follows: m/e 231, ($M^+ - CO_2 + O$), m/e 212, ($M^+ - CO_2 + Cl$), m/e 184, ($M^+ - CO_2 + Cl + CO$), m/e 139 (C_7H_4OCl) and m/e 111 (C_6H_4Cl).

4.1.4. Proton Magnetic Resonance

^{1}H-NMR spectrum of zomepirac obtained from the deuterated DMSO solution is shown on Fig. 4. The spectrum was recorded on JEOL FX-loo spectrometer using TMS as internal standard. Assignements, chemical shifts and relative intensities of signals are presented on Table 1.

Table 1. ^{1}H-NMR spectrum of zomepirac - chemical shifts, relative intensities and assignements



chem. shift (ppm)	intens.	multiplicity	assignement
1.67	3 H	singlet	1
3.67	3 H	singlet	2
3.74	2 H	singlet	3
5.97	1 H	singlet	4
7.59	4 H	multiplet	5
12.66	1 H	broad	6

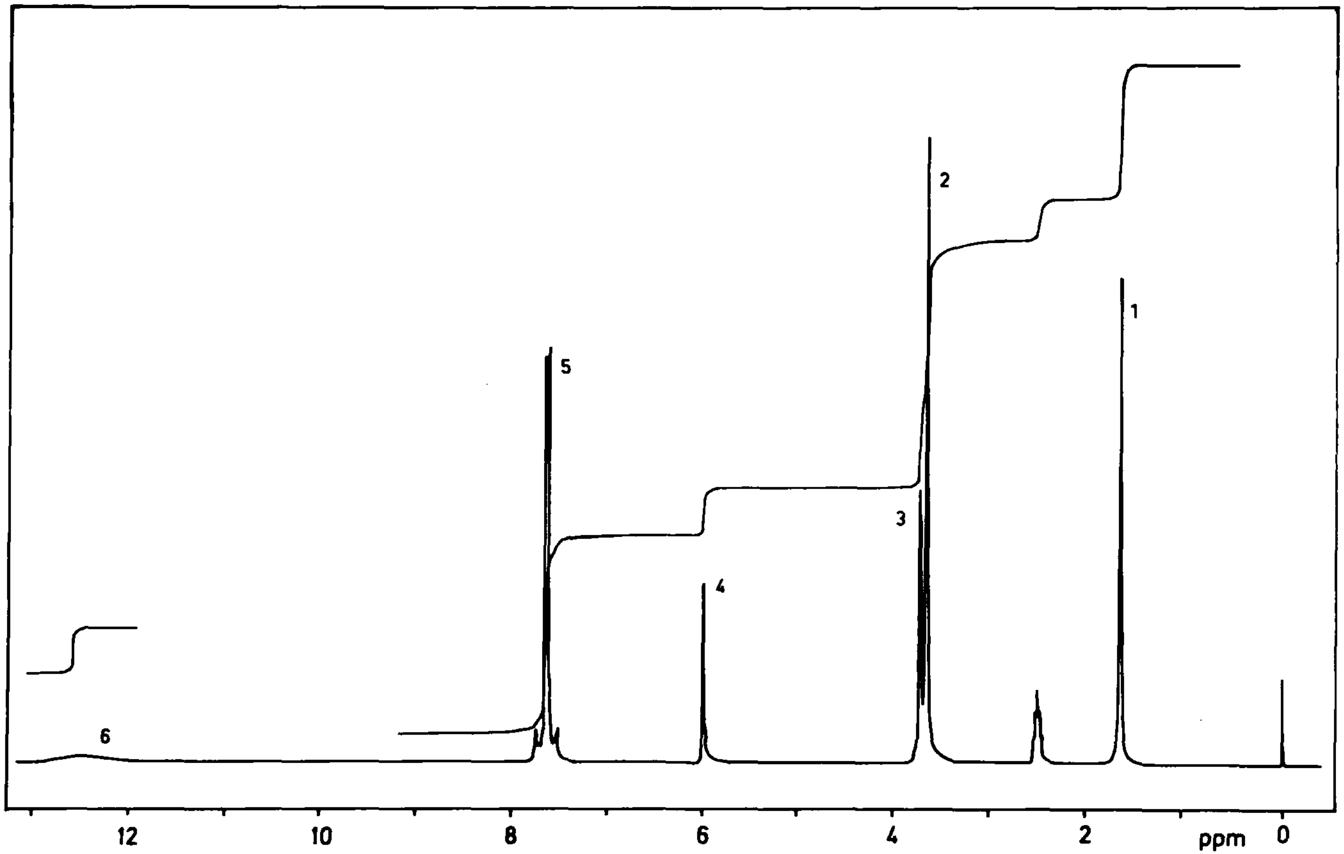


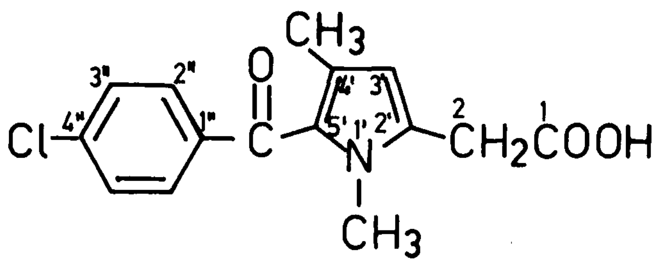
Fig. 4. Proton magnetic resonance spectrum of zomepirac in DMSO-d_6. Instrument: Jeol FX-1oo.

4.1.5. ^{13}C-Nuclear Magnetic Resonance

^{13}C-NMR spectrum of zomepirac shown on Fig. 5 was obtained from the deuterated DMSO solution using TMS as internal standard. It was recorded on JEOL FX-loo spectrometer at 25.o5 MHz. Chemical shifts and coupling constants are given in Table 2.

The assignements for the two methyl, methylene, carboxyl and carbonyl carbons are straightforward because of their well defined regions of chemical shifts. On the other hand, resonances due to benzene and pyrrole rings give a rather complicated pattern in the appropriate region. The additivity rules for substituted benzenes /7/ have

Table 2. ^{13}C-NMR chemical shifts δ^{a} and ^{13}C-^{1}H coupling constants $^{n}J^{b}_{CH}$ for zomepirac



C-atom	δ (ppm)		$^{1}J_{CH}$	$^{2}J_{CH}$	$^{3}J_{CH}$
2'	136.25	s	-	7.9 C-2 8.2 C-3	-
3'	112.15	d	171.2	-	4.3
4'	127.89	s	-	3.3	7.1
5'	128.62	s	-	-	-
2	32.11	t	129.4	-	-
1	170.78	s	-	7.6	-
$N-CH_3$	32.80	q	139.8	-	-
CH_3	14.13	q	127.o	2.4	-
C=O	185.o3	s	-	-	1.8
1"	134.81	s	-	3.7	-
2"	13o.3o	d	164.8	5.5	-
3"	128.45	d	168.5	4.o	-
4"	139.34	s	-	1.o	6.1

[a] δ in ppm downfield from the internal TMS. Off-resonance multiplicities are given an a second dolumn.

[b] J in Hz.

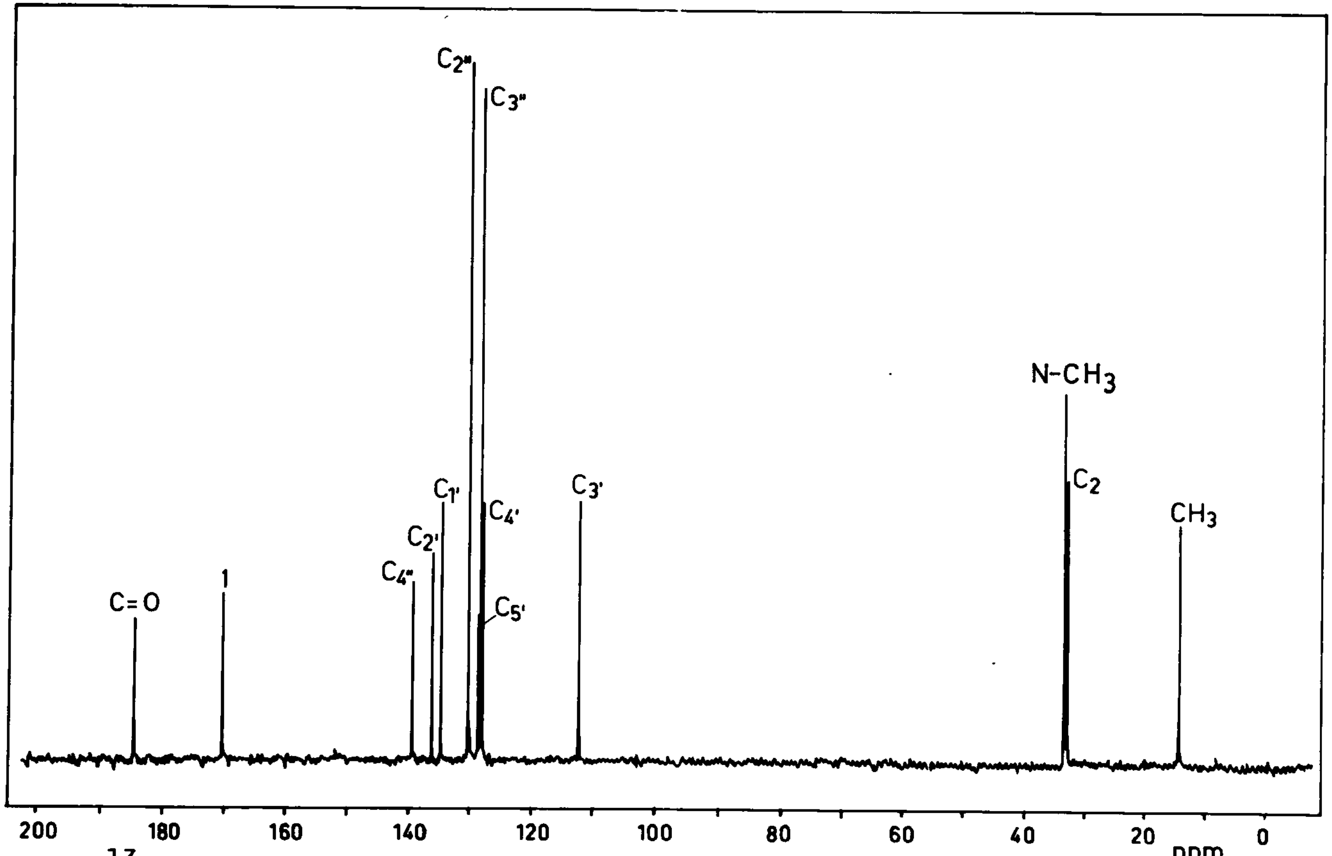


Fig. 5. ^{13}C-NMR broad-band decoupled spectrum of zomepirac. Instrument: Jeol FX-1oo.

helped to assign the signals for C-1", C-2", C-3" and C-4" at 134.81, 130.30, 128.45 and 139.34 ppm, respectively. The off-resonance spectrum, and the comparison with the related spectrum of p-chloroacetophenone /8/, confirmed this assignement. However, the pyrrole ^{13}C-resonances were not easy to assign because of the complex substituent effects. In a gated decoupled spectrum the signal at 112.15 ppm was a doublet with a characteristic first-order $^{13}C-^{1}H$ coupling constant at 171.2 Hz. The other three carbons were assigned from the intricate long-range coupling effects. It should be noted that the C-5' resonance is, surprisingly, situated at higher field (128.62 ppm) than should be expected for a pyrrole ring carbon carrying a carbonyl group /9/.

4.2. Solid Properties

4.2.1. Melting Characteristics

Zomepirac can be conveniently crystallized from isopropanol. The crystalline product needles melts and decomposes at 178-179 °C.

The sodium salt of zomepirac crystallizes as the dihydrate from an isopropanol/water mixture. The salt melts at 295-296 °C.

4.2.2. X-Ray Diffraction

X-Ray diffraction patterns for zomepirac and its sodium salt dihydrate are given in Tables 3 and 4, respectively. Diffraction spectra were produced by monochromatic radiation from the CuK_{α} line (1.542 Å) which was obtained by excitation at 35 kV and 20 mA. Recording conditions were as follows. Optics: detector slit 0.2°, M.R. soller slit 3°, beam slit 0.0007", Ni filter 3° take off angle. Goniometer: scan at 2°, 20/min. Detector: amplifier gain 16 coarse, 9.1 fine. Scintillation counter tube and DC voltage at plateau. Pulse light selection E_L 9V, E_n out. Rate meter: T.C. 1.0, 1000 cps for zomepirac and 1.0, 2000 for sodium salt.

4.3. Solution Properties

4.3.1. Solubility

Zomepirac is freely soluble in methanol, ethanol and hot isopropanol. It is soluble in acetone and chloroform, slightly soluble in ether and practically insoluble in methylenchloride, toluene and hydrocarbon solvents.

4.3.2. Acidity pK_a

pK_a Value of zomepirac was determined by potentiometric titration using an automatic burette, Model ABU 13,

Table 3. X-Ray diffraction data of zomepirac

Θ°	interplanar distance d^a (Å)	rel. intensity I/I_o [b]
4.16	1o.63	6
5.13	8.62	7
5.o8	7.4o	15
7.3o	6.o7	5o
8.24	5.38	57
1o.33	4.3o	75
1o.63	4.18	1oo
11.32	3.93	75
11.6o	3.63	49
12.4o	3.59	16
13.71	3.5o	21
13.o8	3.41	19
13.31	3.53	51
13.98	3.19	25
14.32	3.12	9
15.99	2.8o	6
16.17	2.77	1o
16.58	2.7o	17
17.38	2.58	12
18.42	2.44	14
19.79	2.28	8
21.77	2.o8	1o

[a] $d = n\lambda/2 \sin\Theta$
[b] Based on the highest intensity which is selected as unity.

coupled with the recording unit Titrigraph SBR-2C of a Titrator TTT2 (all equipment from Radiometer-Copenhagen). The glass electrode G-2o2 c, was used against a calomel K 4o1 reference electrode. Zomepirac accurately weighed and dissolved in an ethanol/water mixture 1:1(v/v), was titrated with o.1 M NaOH solution. Potentiometric curves were recorded between pH 3.45-12.o and the pK_a value of 4.75 was obtained by calculation according to ref. /1o/.

5. Methods of Analysis

5.1. Elemental Analysis

Percentages of elemental content of a zomepirac molecule and its sodium salt dihydrate are given in Table 5.

Table 4. X-Ray diffraction data of zomepirac sodium salt dihydrate

$\Theta°$	interplanar distance d^a (Å)	rel. intensity I/I_o [b]
6.07	7.29	11
7.00	6.33	32
7.64	5.80	84
8.10	5.48	65
8.34	5.31	28
9.11	4.87	31
9.89	4.49	32
10.50	4.23	71
10.92	4.07	100
11.64	3.82	20
11.85	3.75	30
12.68	3.51	44
13.90	3.21	48
14.23	3.14	30
15.37	2.91	16
15.60	2.87	12
16.45	2.72	19
16.06	2.64	10
17.31	2.59	20
17.59	2.55	21
20.00	2.15	14
21.41	2.11	13
21.90	2.07	25
24.91	1.83	14

[a,b] See footnotes in Table 3.

Table 5. Elemental composition of zomepirac and its sodium salt dihydrate

	Mol. formula	% of the element (theor.) C	H	Cl	N	Na	O
zomepirac	$C_{15}H_{14}ClNO_3$	61.79	4.89	12.16	4.80	-	16.41
sodium salt x x 2 H_2O	$C_{15}H_{13}ClNNaO_3$ x 2 H_2O	51.51	4.89	10.14	4.00	6.57	22.89

5.2. Chromatographic Methods

5.2.1. Thin Layer

R_f values and solvent systems for eluation of zomepirac on Merck F_{254} silicagel plates are given in Table 6. Spots are located under an UV_{254} lamp.

Table 6. R_f values and solvent systems for TL chromatography of zomepirac

solvent system	R_f
MeOH	o.78
CH_2Cl_2/conc. HOAc 3:1	o.8o
EtOAc/conc. HOAc 6:1	o.82
ether/conc. HOAc lo:1	o.84
$CHCl_3$/conc. HOAc lo:1	o.62
cyclohexane/conc. HOAc 8:1	o.12

5.2.2. Gas

Zomepirac was chromatographed on coiled glass column 1 m x 4 mm i.d. filled with Gas Chrom Q 8o-1oo mesh impregnated with 3% OV-1o1. Temperature maintenance was as follows: oven at 2oo°C, injection block at 3oo°C, detector at 3oo°C. The gas rates (ml/min) were: carrier gas (N_2) and hydrogen 4o, air 4oo. The column efluent was monitored with a flame-ionisation detector. Zomepirac was injected in the form of its methyl ester which was obtained by the following procedure: 1o mg of zomepirac was mixed with o.3 ml of 1o% methanolic BF_3 solution, heated at 6o°C for 1o min and allowed to cool. The resulting liquid was mixed with 3 ml of hexane, transfered to a separatory funnel and washed three times with a saturated aqueous sodium sulphate. The dry liquid was used directly for GC. Gas chromatogram of zomepirac methyl ester is shown on Fig. 6.

Alternatively, zomepirac can be esterified by diazomethane solution; if it is analysed without esterification decarboxylation takes place during the GC analysis /11/.

5.2.3. High Performance Liquid

Zomepirac can be analysed by HPLC on a column filled with Supelcosil LC-8 (5 µm). As a mobile phase the mixture of acetonitrile, o.o5 M KH_2PO_4 and phosphoric acid (85%, d=1.71), 6o, 4o and 2 ml, respectively, was used; the mixture had pH value of 3.5. The mobile phase was forced through the column at 4o-5o bar; this pressure maintained a flow rate of 1.7 ml/min. The efluent was monitored at 323 nm. HPL chromatogram is shown on Fig. 7.

5.3. Titration

Thirty mg of zomepirac was weighed (±o.1 mg) into a titration vessel and dissolved in a mixture of 1o µl of ethanol and 1o ml of water. The resulting solution was

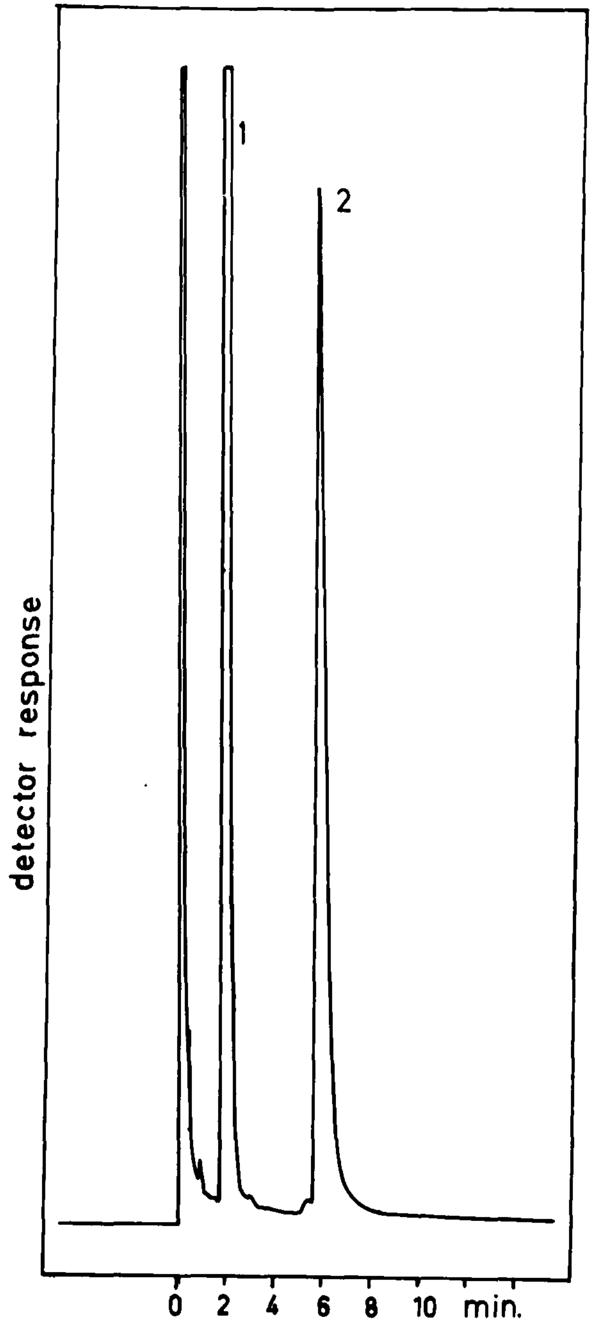


Fig. 6. Gas chromatogram of zomepirac methyl ester (peak 2) and internal standard methyl heptanoate, (peak 1). Instrument: Pye-Unicam 2o4 chromatograph.

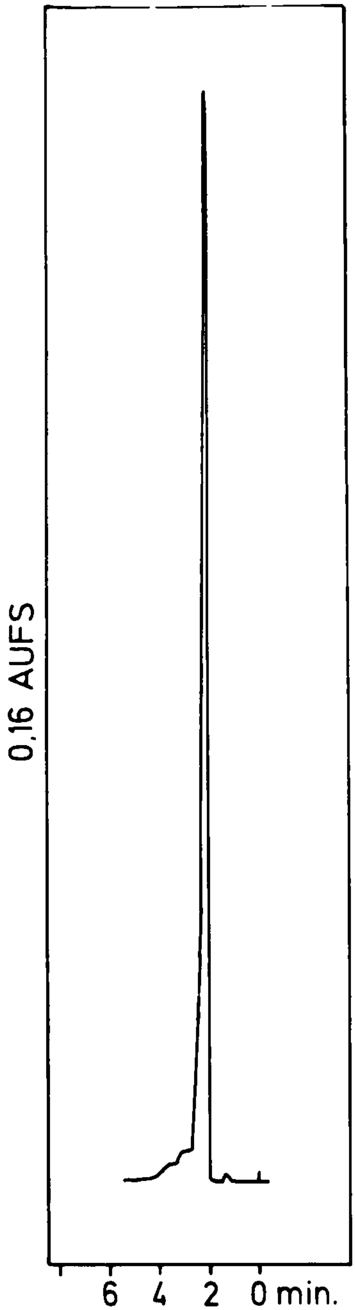


Fig. 7. HPL chromatogram of zomepirac. Instrument: Pye-Unicam LC-3-XP.

titrated potentiometrically using the glass and calomel electrode pair. The content of zomepirac was calculated from the quantity of consumed o.1 M NaOH solution using the equation (1):

$$\text{content of zomepirac } \% = \frac{V \times f \times E}{N} \times 100 \qquad (1)$$

where V = volume of o.1 M NaOH solution consumed (ml)
f = normality factor of o.1 M NaOH solution
E = 29.16 - equivalent of zomepirac for 1 ml of o.1 M NaOH solution
W = mass of the sample (mg)

Using the above procedure, the solution of 5o mg of zomepirac sodium salt dihydrate in 2o ml of water was titrated with o.1 M hydrochloric acid solution. The content of the zomepirac sodium salt dihydrate was calculated using the equation (1) where V = volume of o.1 M hydrochloric acid consumed (ml), f = normality factor of o.1 M hydrochloric acid solution and E = 33.96, equivalent of zomepirac sodium salt dihydrate for 1 ml of o.1 M hydrochloric acid solution.

6. Stability and Degradation

Zomepirac and its sodium salt dihydrate, powdered, and tablets containing sodium salt dihydrate were kept at 4o-5o % relative humidity, at temperatures within the range of 45-5o °C for seven days. The samples were analysed by HPLC. Tablets and powdered salt dihydrate did not show any change but 7.6% of nonidentified impurity appeared in the powder of zomepirac.

7. Drug Metabolism, Pharmacokinetics, Bioavailability

Because of its lipophilicity, zomepirac may owe at least part of its analgesic activity to an increased ability to penetrate into the spinal cord and brain and subsequently to the inhibition of prostaglandin synthesis within the central nervous system /12/.

On the basis of urinary recovery data, zomepirac is almost absorbed after oral administration of dosages of 25-2oo mg to healthy subjects. Mean peak plasma concentrations of 2.47, 4.42 and 7.94 μg/ml were attained 44, 57 and 8o min after a single oral dose of 5o, 1oo or 2oo mg, respectively. Bioavailability of zomepirac is the same after ingestion of tablets, capsules or an aqueous solution. Zomepirac is extensively bound to plasma albumin (98.5%) in man. Maximal plasma concentrations (4ooo ng/ml) are reached in 1 hour at dose of 1oo mg.

Tissue concentrations of radiolabelled zomepirac in

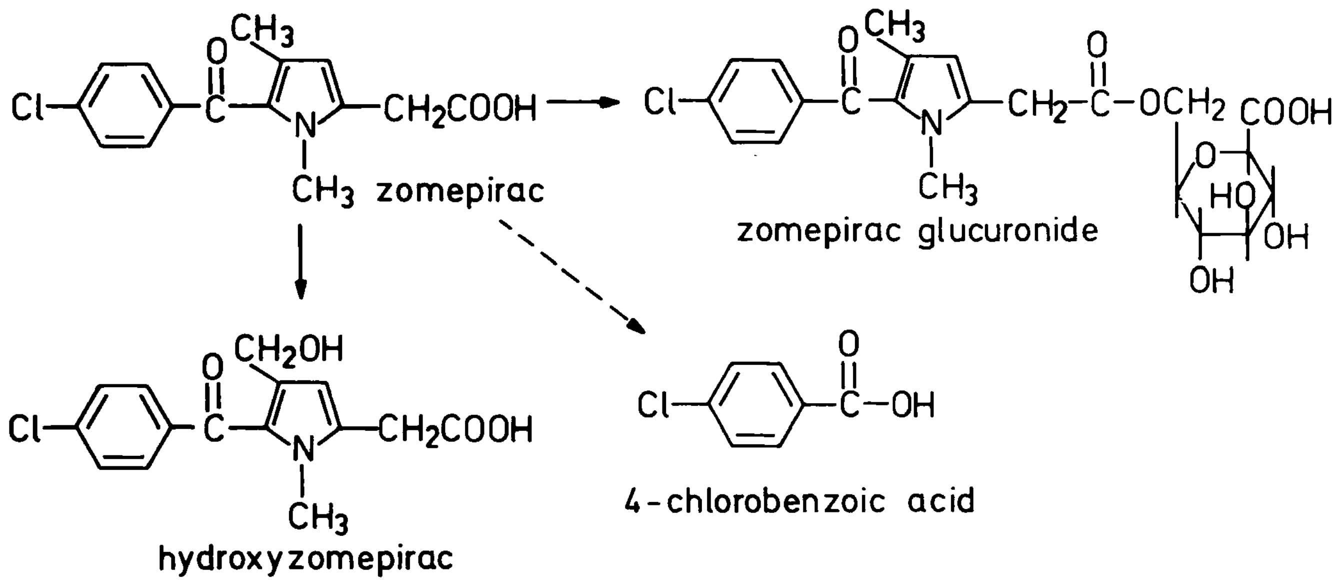


Fig. 8. Metabolites of zomepirac.

rats are in the stomach, kidneys, intestine, and liver, 2.9, 2.2, 1.7, and o.5 times, respectively, plasma concentration 6 hours after oral administration of a 6 mg/ /kg dose. The rat plasma concentrations of zomepirac and metabolites are about 5 times the tissue concentrations of highly perfused organs such as the heart and lungs, and about 5o times that of the brain. After 48 hours about o.3% of the dose remains in the carcass of the rat, o.1% of the dose being concentrated in the liver /13/. Zomepirac is detected in the cerebrospinal fluid of cats 1o minutes after intravenous administration of a 3 mg/kg dose. Cerebrospinal concentrations were approximately 7% of plasma concentrations 24 hours after dosing /14/.

The elimination half-life of zomepirac is about 4 hours following a single dose, but may be increased following multiple doses. Plasma clearance after doses of 4o mg/kg in rhesus monkeys is depressed to less than one half that observed after doses of 5 and 1o mg/kg. Authors consider that the reason for this nonlinear kinetics is saturation of metabolic conjugation /15/.

Zomepirac is excreted almost exclusively in the urine, the major metabolite being the glucuronide conjugate, which accounts for about 57% of radioactivity after a 25 mg dose. Hydroxyzomepirac and 4-chlorobenzoic acid are minor metabolites /Fig. 8/ and they are approximately 2oo-3oo times less active than zomepirac as inhibitors of human platelet aggregation in vitro. About 22% of the dose is excreted in urine unchanged.

8. Identification and Determination in Body Fluids and Tissues

The determination of zomepirac in the blood, plasma and urine is possible by HPL and gas chromatographic methods /11,16,17/. HPL chromatographic determination of zomepirac in the human plasma allows its determination at concentrations down to 1o ng/ml for 2 ml plasma samples and 5o ng/ml for 1 ml plasma samples /16,17/. Schütz and Suphachearabhan /11/ reported that UV photometry seemed to be the best method for the urine screening. The determination of zomepirac and its metabolites by a contionous body fluid monitoring system based on HPLC was described by Müller and Zulliger /18/. Their method allows a determination of zomepirac down to 5o ng/ml in the 5o μl/ml plasma samples; in the concentration range of o.5-1oo μg/ml of the plasma a full linearity was achieved with a recovery of 97% for the plasma samples and 95% for the urine samples.

In the references /11/ and /19/ reversed phase HPLC

was used under the following operating conditions:

Column: Spherisorb ODS, 5 μm (125 mm x 4.9 mm), Kontron AG

Mobile phase: acetonitrile, water, orthophosphoric acid 5o : 49.5 : o.5 ml, respectively.

Flow rate: 2 ml/min.

Detector: UV, 313 nm.

Sensitivity: variable

A more sensitive and precise method for the determination of zomepirac in the human plasma is GC /2o/. In this method, zomepirac must be transformed in its pentafluorobenzyl ester prior to GC analysis. The use of an electron capture detector (ECD) allows the determination of zomepirac pentafluorobenzyl ester even in the picogram range. The lowest accurate concentration is 5 ng/ml for 2 ml plasma samples and 25 ng/ml for o.1 ml plasma samples. Column filling and operating maintenance were as follows:

Column: 122 cm x o.4 cm i.d., sililated glass filled with a Gas Chrom Q (6o-8o mesh) impregnated with 3% OV-17. Temp. 23o °C.

Carrier gas: argon with 5% of methane.

Detector: ECD, ^{63}Ni. Temp. 295 °C.

Injector: 2oo °C.

A combination of GC and MS allows identification and determination of zomepirac and its metabolites in body fluids /11,2o/.

9. Determination in Pharmaceuticals

Preparing an anlytical solution: ten tablets, each containing 119.89 mg of zomepirac sodium salt dihydrate, are powdered in a mortar and a mass of powder equivalent to 15o mg of zomepirac is weighed into a 1oo ml volumetric flask. Fifty ml of water are added, the content shaken for 3o min, and the volume made up to mark. An aliquot of 15 ml is centrifugated (3ooo g, 1o min), and the supernatant taken for analysis (solution A).

Standard solution: into a 1o ml volumetric flask 18.o mg of zomepirac sodium salt dihydrate are weighed in, dissolved in water and the volume made up.

Work up and measurement: 2 ml of clear solution A are pipetted into a 1o ml test tube. To each tube are added: 1 ml of o.1 N HCl solution and 5 ml of ether containing o.5 mg/ml of methyl heptanoate as an internal standard. Both tubes are stoppered, shaken for 5 min. and left to stand for another 5 min. Thereafter 2 ml of the ethereal layers were transfered into two clean test-tubes and the ether was completely evaporated in a stream of air. To each residue were added 2 ml of 1o% methanolic boron tri-

fluoride solution. Both tubes were then placed in block-thermostat, kept at boiling temperature for 5 min, then left to cool down to room temperature. After addition of 8 ml of water and 1 ml of hexane the methyl ester of zomepirac is extracted into the hydrocarbon layer and the extract injected into a gas chromatograph. Determination of the detector relative weight response (RWR): one µl of the hexane extract from the standard was injected into the chromatograph. The RWR value was calculated according to equation (2):

$$RWR = \frac{A_z \times C_{I.S.}}{A_{I.S.} \times C_z} \qquad (2)$$

where A_z = peak area of zomepirac methyl ester, $A_{I.S.}$ = = peak area of internal standard methyl heptanoate; C_z= = concentration of zomepirac methyl ester in mg/ml, $C_{I.S.}$ = = concentration of methyl heptanoate in mg/ml

Content of zomepirac in one tablet: the hexane extract of the sample solution was injected into the gas chromatograph and the desired content was calculated using equation (3):

$$\text{mg of zomepirac per tablet} = \frac{A_z \times C_{I.S.} \times 5o \times W_a}{A_{I.S.} \times RWR \times W} \qquad (3)$$

where A_z, $A_{I.S.}$ and $C_{I.S.}$ have the same meaning as in eq. 2 , W_A is the average mass of a tablet and W = mass of the powdered sample /21/.

Acknowledgement The authors are thankful to dr A. Nagl, Laboratory of General and Inorganic Chemistry, Faculty of Science, University of Zagreb, for X-ray diffraction data.

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PROFILE SUPPLEMENTS

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CHLORAMPHENICOL

Abdullah A. Al-Badr and Humeida A. El-Obeid

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

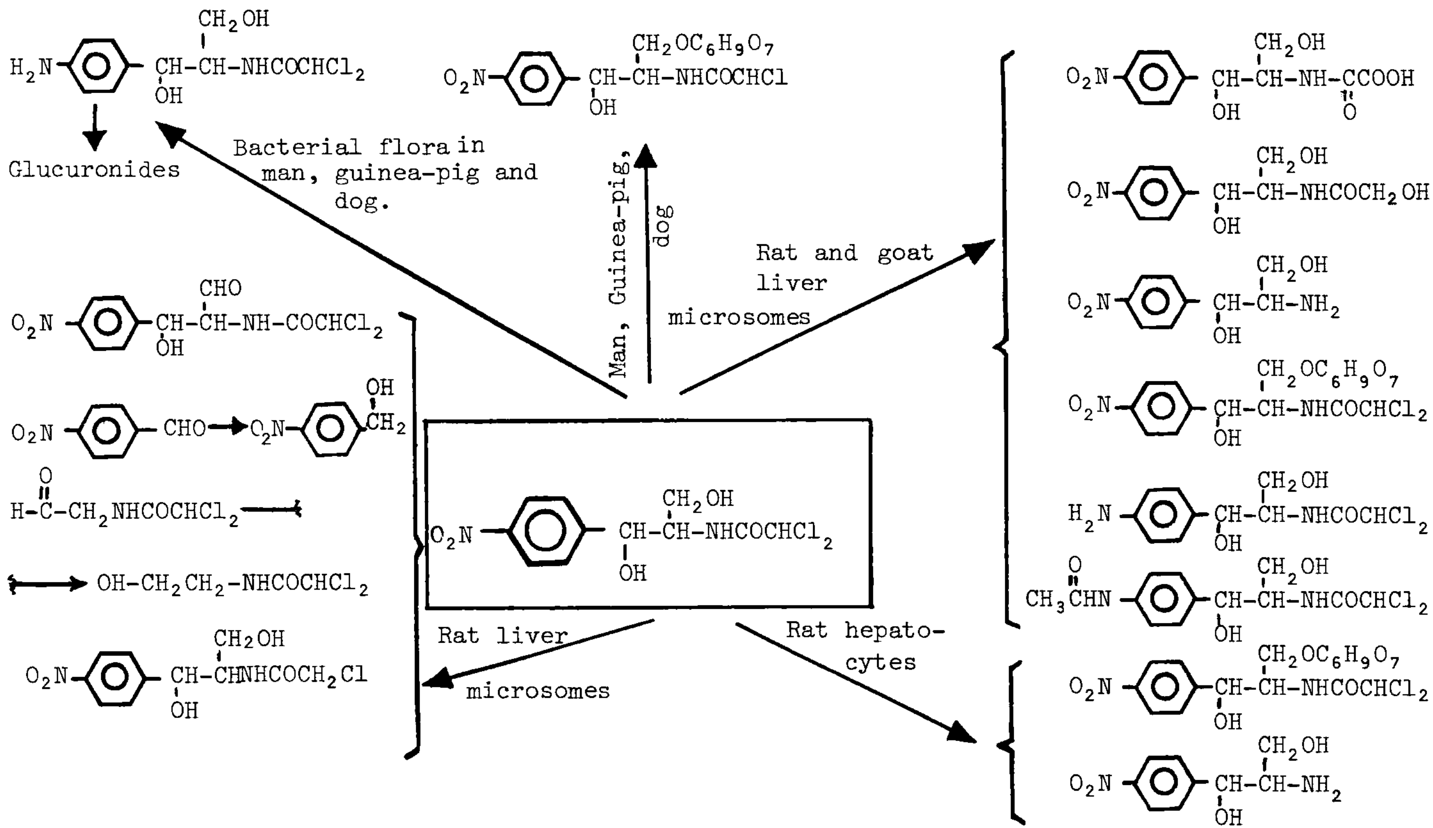
D(-)-threo-2-Dichloroacetamido-1-p-nitrophenyl-1,3-propanediol.
D(-) threo-N-Dichloroacetyl-1-p-nitrophenyl-2-amino-1,3-propanediol.
D(-) threo-2,2-Dichloro-N-[β-hydroxy-α-(hydroxymethyl)-p-nitrophenethyl]acetamide.
D(-) threo-2,2-Dichloro-N-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]acetamide.

1.1.2 Generic Names

Chloramphenicol, Chloramfenicol, Chloramphenicolum, Chloramphenicolo, Laevomycetin.

1.1.3 Trade Names (1)

Alficetyn, Ambofen, Amphicol, Anacetin, Aquamycetin, Bemacol, Berlicetin, Biocetin, Biophenicol, Cafenolo, Cebenicol, Chemicetina, Chemyzin, Chlomin, Chloramex, Chloramol, Chloramphenicol-POS, Chlorasol, Chlora-tabs, Chloricol, Chlornitromycin, Chlorocid, Chloromycetin, Chloronitrin, Chloroptic, Chloro-25 Vetag, Chlorsig, Cloramidina, Clorbiotina, Clorofenicina, Cloromicetin, Cloromycetin, Clorosintex, Comycetin, Cylphenicol, Desphen, Detreomycine, Devamycetin, Dextromycetin, Doctamicina, Duphenicol, Econochlor, Erbaplast, Ertilen, Farmicetina, Fenicol, Globenicol, Glorous, Halomycetin, Hortfenicol, Isicetin, Ismicetina, Isophenicol, Isopto Fenicol, Kamaver, Kemicetin, Kemicetine, Kloromisin, Labamicol, Leukomycin, Levomycetin, Lomecitina, Loromisin, Mammaphenicol, Medichol, Micochlorine, Misetin, Mycetin, Mychel, Mycinol, Neocetin, Novochlorocap, Nova-Phenicol, Novophenicol, Oftakloram, Oftalent, Oleomycetin, Opclor, Ophtaphenicol, Ophtochlor, Oralmisetin, Otachron, Otomycin, Otophen, Pantovernil, Paraxin,



Scheme 4. Metabolites of chloramphenicol in *in vivo* and *in vitro* studies.

1.2.3 CAS Registry No.

[56-75-7]

1.2.4 Wiswesser Line Notation

WNR DYQYIQ MVYGG (2).

1.3 Molecular Weight

323.13, 322.01 (2)

1.4 Elemental Composition

C 40.88%, H 3.74%, Cl 2-1.95%, N 8.67%, O 24.76%.

1.5 Appearance, Color, Odor and Taste

Fine white to greyish white or yellowish white crystals, needles or elongated plates from water or ethylene dichloride with very bitter taste.

2. Physical Properties

2.1 Melting Point

150.5° - 151.5°.

2.2 Solubility

Soluble (25°) in water : 2.5 mg/ml, in propylene glycol : 150.8 mg/ml, very soluble in methanol, ethanol, butanol, ethyl acetate, acetone. Fairly soluble in ether, insoluble in benzene, petroleum ether, vegetable oils. Solubility in 50% acetamide solution is 5%. Aqueous solutions are neutral. Neutral and acid solutions are stable on heating.

2.3 Spectral Properties

2.3.1 Ultraviolet Spectrum

The ultraviolet absorption spectrum of chloramphenicol in neutral methanol was obtained on a Cary 219 spectrophotometer. The spectrum, shown in Fig. 1, is characterized by a maximum at 274 nm and a minimum at 235 nm. The spectrum of chloramphenicol in

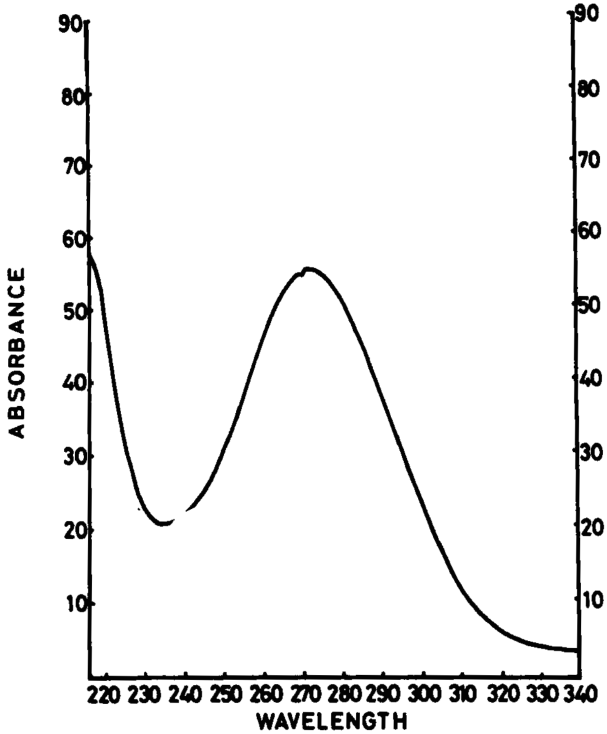


Figure 1. Ultraviolet spectrum of chloramphenicol in neutral methanol.

water showed a maximum at 278 nm (E 1%, 1 cm 298); in 0.1N NaOH, a maximum at 276 nm (E 1%, 1 cm 200); in 0.1N H_2SO_4, a maximum at 278 nm (E 1%, 1 cm 284) (3).

2.3.2 Infrared Spectrum

The infrared absorption spectrum of chloramphenicol obtained from a potassium bromide dispersion is shown in Figure 2. The spectrum was recorded on a Pye Unicam SP 1025 infrared spectrophotometer. The characteristic bands of the spectrum with the assignments are listed below:

Frequency (cm^{-1})	Assignment
3230, 3520	Broad H-bonded OH and NH stretch
3100	Aromatic C-H stretch
1700, 1570	C = O stretch amide 1 band, amide II band.
1530, 1360	N $\overset{\cdot\cdot\cdot}{—}$ O stretch ($ArNO_2$)
1070	C - O stretch (primary alcohol)
850	C - N stretch ($ArNO_2$)

The principal peaks as reported by Clarke (3) are 1682, 1061 and 1351 or 1526 cm^{-1}

2.3.3 ^1H-Nuclear Magnetic Resonance Spectroscopy (^1H NMR)

^1H NMR spectra of chloramphenicol in DMSO-d_6 (Figure 3) and in DMSO-d_6 in the presence of D_2O (Figure 4) are obtained on a Varian-T60A NMR spectrometer. The band assignments are referenced to TMS and are listed below:

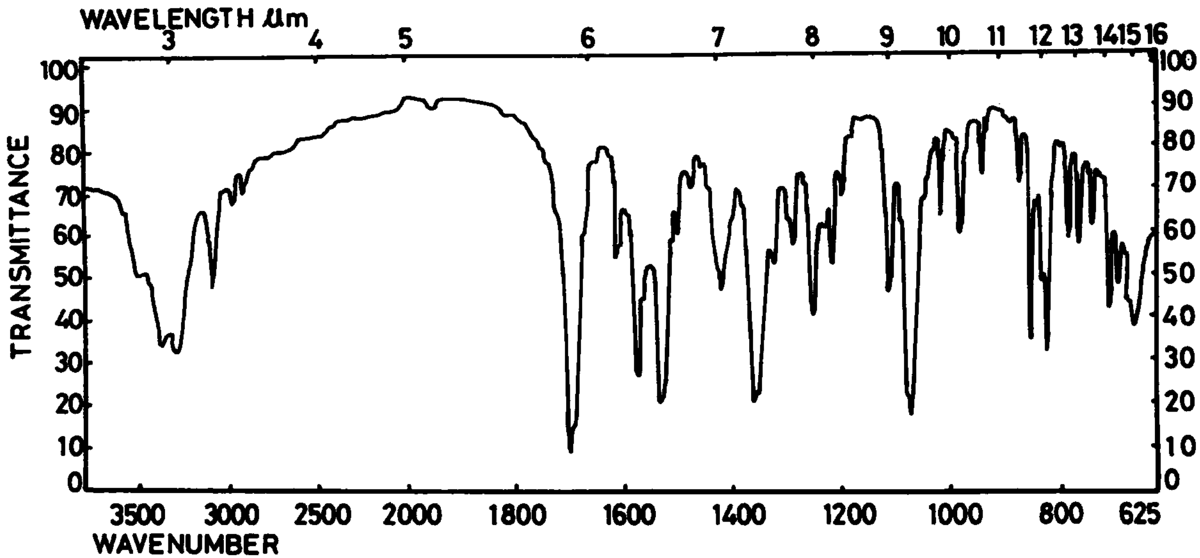


Figure 2. Infrared spectrum of chloramphenicol from KBr disc.

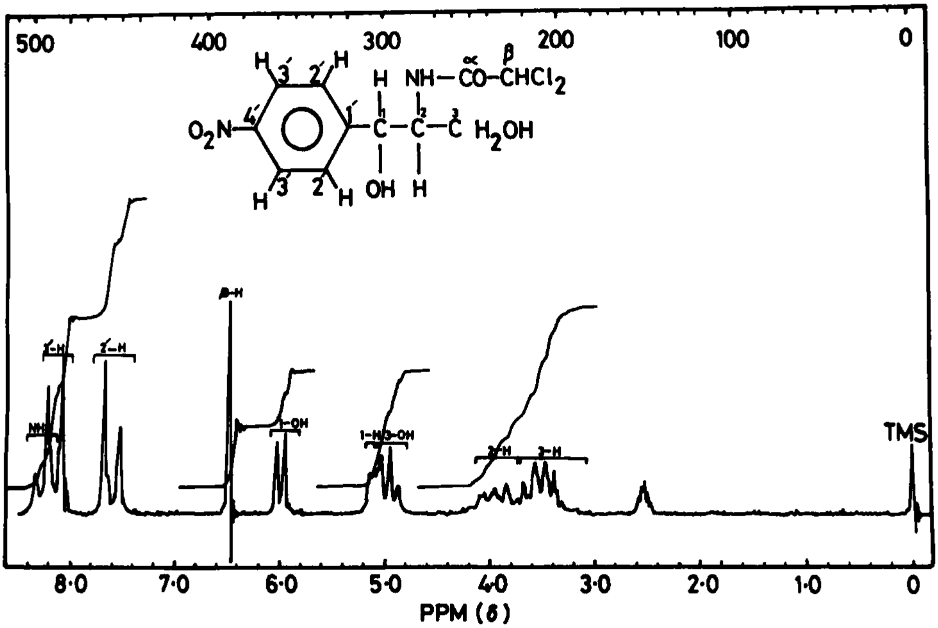


Figure 3. ^{1}H-NMR spectrum of chloramphenicol in DMSO-d_6.

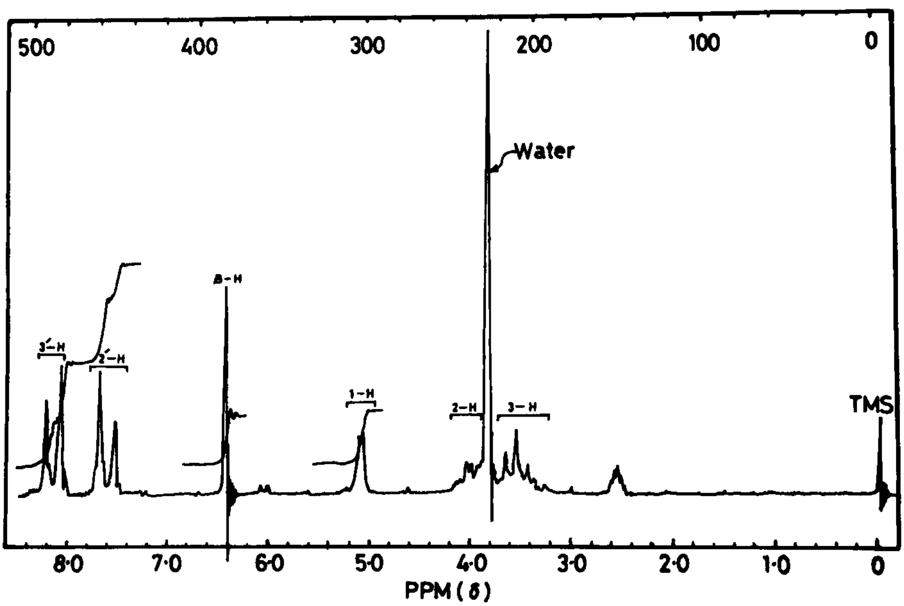
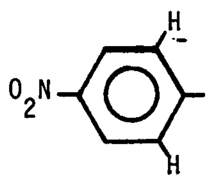
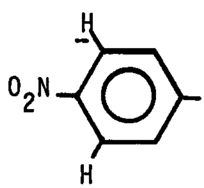


Figure 4. ^{1}H NMR spectrum of chloramphenicol in DMSO-d_6 and D_2O.

Chemical shift* (δ)	Multiplicity	Proton Assignment	No. of Protons
a. 3.43 b. 3.47	Multiplet	$-\underline{CH_2}OH$	2
a. 3.97 b. 3.98	Multiplet	$C\underline{H}$-N-	1
a. 4.93	Triplet	$-CH_2-O\underline{H}$	1
b. Exchanged	-	-	-
a. 5.12 b. 5.08	Triplet	$C\underline{H}$-OH	1
a. 5.97	Doublet	CH-$O\underline{H}$	1
b. Exchanged	-	-	-
a. 6.47 b. 6.40	Singlet	$-COC\underline{H}Cl_2$	1
a. 7.60 b. 7.58	Doublet		2
a. 8.15 b. 8.13	Doublet		2
a. 8.25	Doublet	-NH	1
b. Exchanged	-	-	-

* a, in DMSO-d_6 ; b, in DMSO-d_6 + D_2O.

2.3.4 ^{13}C Nuclear Magnetic Resonance (^{13}C NMR)

The ^{13}C NMR spectra of chloramphenicol are obtained in DMS-d_6, containing a drop of $CDCl_3$, at ambient temperature with ^1H-decoupling (Figure 5) and off-resonance (Figure 6). The spectra are recorded using TMS as internal standard on a JEOL FX100 MHz instrument. The chemical shifts, multiplicities and spectral assignments are given below:

Chemical shift (δ)	Multiplicity	Carbon assignment*
56.71	Doublet	C_2
60.18	Triplet	C_3
66.35	Doublet	C_1
68.93	Doublet	C_β
122.76	Doublet	C_2'
127.17	Doublet	C_3'
146.31	Singlet	C_1'
151.12	Singlet	C_4'
163.33	Singlet	C_α

*Refer to structure in Figure 5 for carbon numbering.

2.3.5 Mass Spectrum

The combined gas-chromatographic/mass-spectrophotometric technique was used for the identification and analysis of chloramphenicol in aqueous solutions (4) and for chloramphenicol and its metabolites in animal tissues and body fluids (5,6). Becker *et al* (7) and Krueger (8) studied the spectra of non-volatile substances by fission fragment

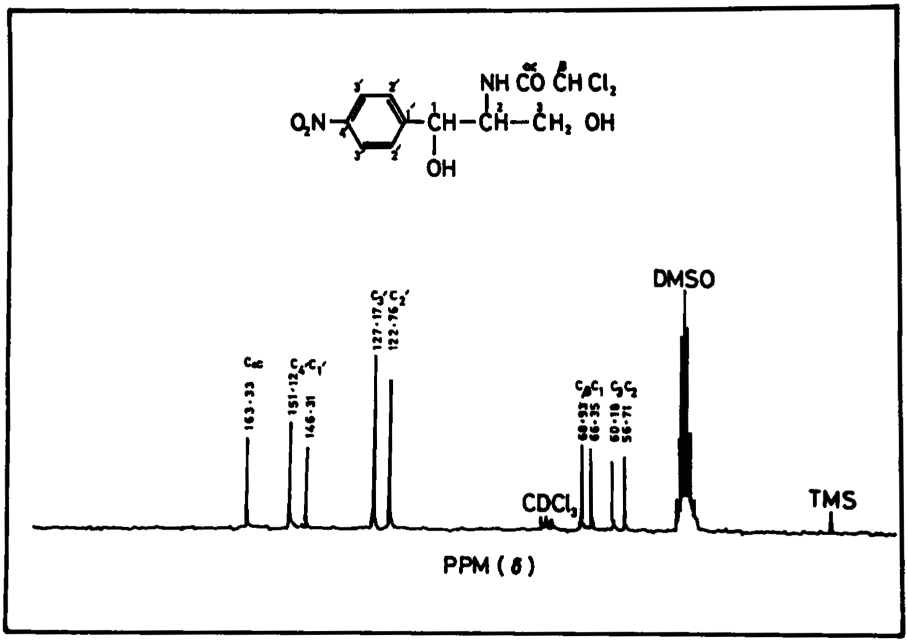


Figure 5. Proton decoupled ^{13}C NMR spectrum of chloramphenicol in DMSO-d_6 + a drop of $CDCl_3$.

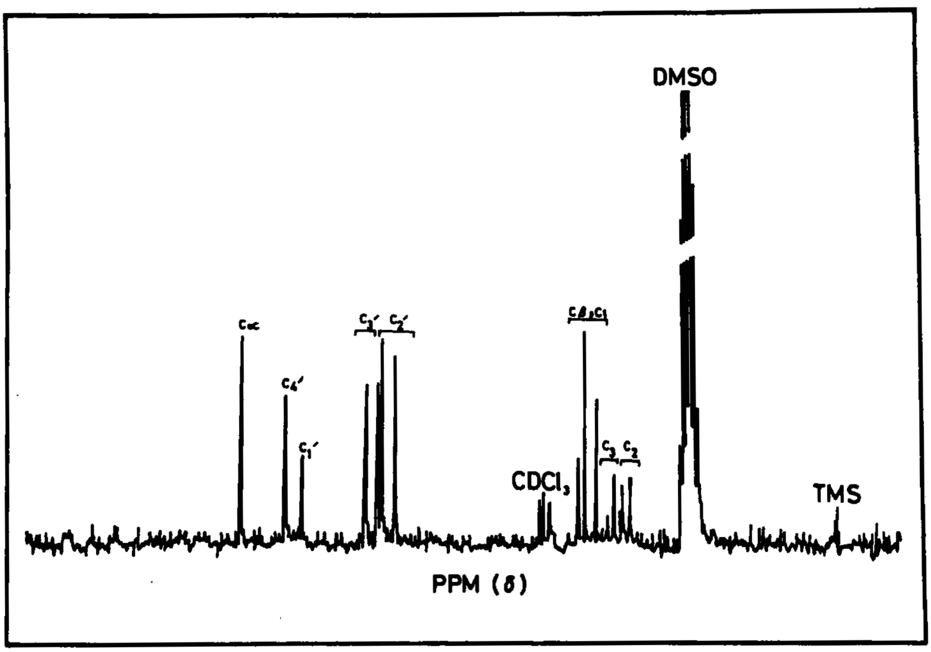


Figure 6. Off-resonance ^{13}C NMR spectrum of chloramphenicol in DMSO-d_6 + a drop of $CDCl_3$.

desorption mass spectrometry. In the spectra of chloramphenicol both quasimolecular ions are presented with their typical two-chlorine isotopic pattern. The positive ion spectra show various different subgroups. Although there is very little fragmentation in the negative ion spectrum, a strong peak group arises near m/e 152.

El spectrum of chloramphenicol was shown (2) to possess the following peaks: 153(100%), 60(99%), 70(85%), 155(78%), 170(70%), 106(46%), 77(40%).

Presented in Figure 7 is the 70 eV electron impact (EI) mass spectrum of chloramphenicol obtained on Varian MAT 311 mass spectrometer using ion source pressure of 10^{-6} Torr, ion source temperature of 180°C and an emission current of 300 µA. No molecular ion is detected and the spectrum is dominated by m/e 153 ion (base peak) resulting from the loss of $O_2NC_6H_4CHO$ and H_2O. A proposed mechanism of fragmentation and the mass/charge ratios of the major fragments is given in Scheme 1. The chemical ionization (CI) spectrum (Figure 8) with methane gas as a reagent is obtained on a Finnigan 4000 mass spectrometer with ion electron energy of 100 eV, ion source pressure of 0.3 Torr, ion source temperature of 150°C and emission current of 300 µA. The spectrum shows no parent molecular ion but a pronounced peak resulting from the loss of water (MH^+-18) constitute the base peak at m/e = 305. A quasi-molecular ion (M + 1) is also prominent. Two peaks appearing at m/e = 351 and m/e 363 are attributed to the transfer of carbocations from the carrier gas. The mass spectral assignment of the prominent ions under CI conditions is given in Table I.

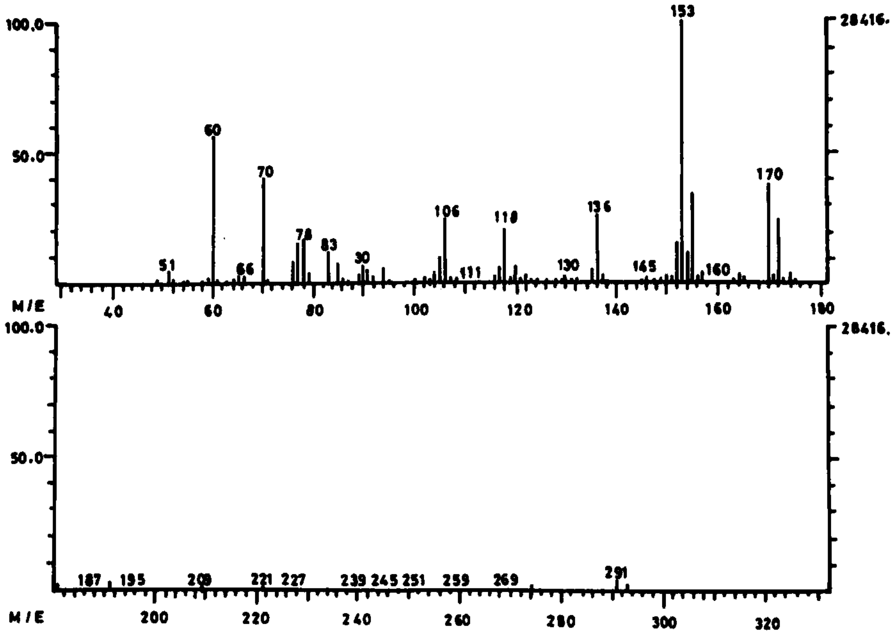


Figure 7. Mass spectrum of chloramphenicol (EI).

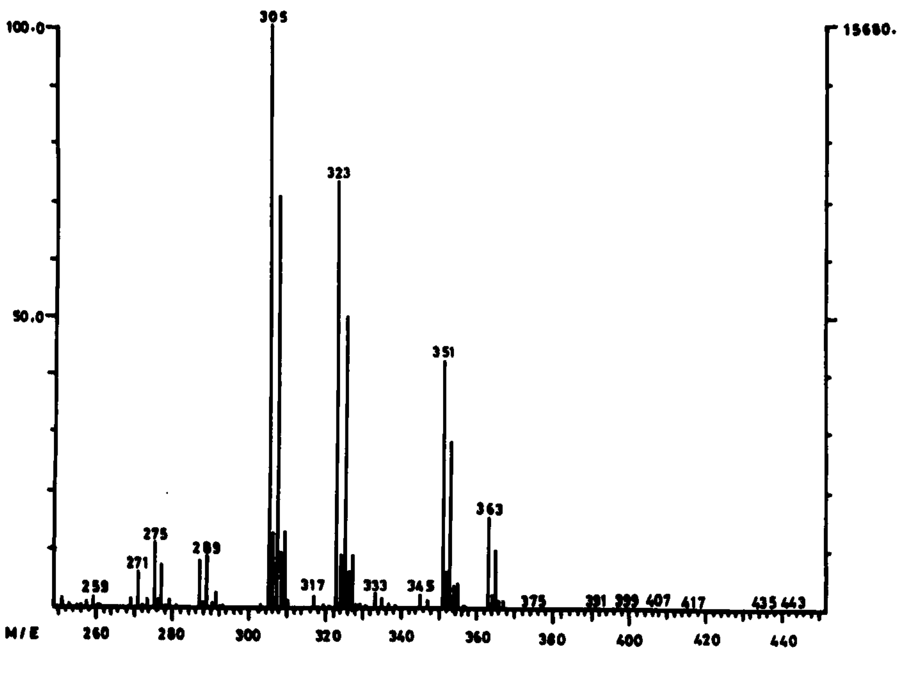
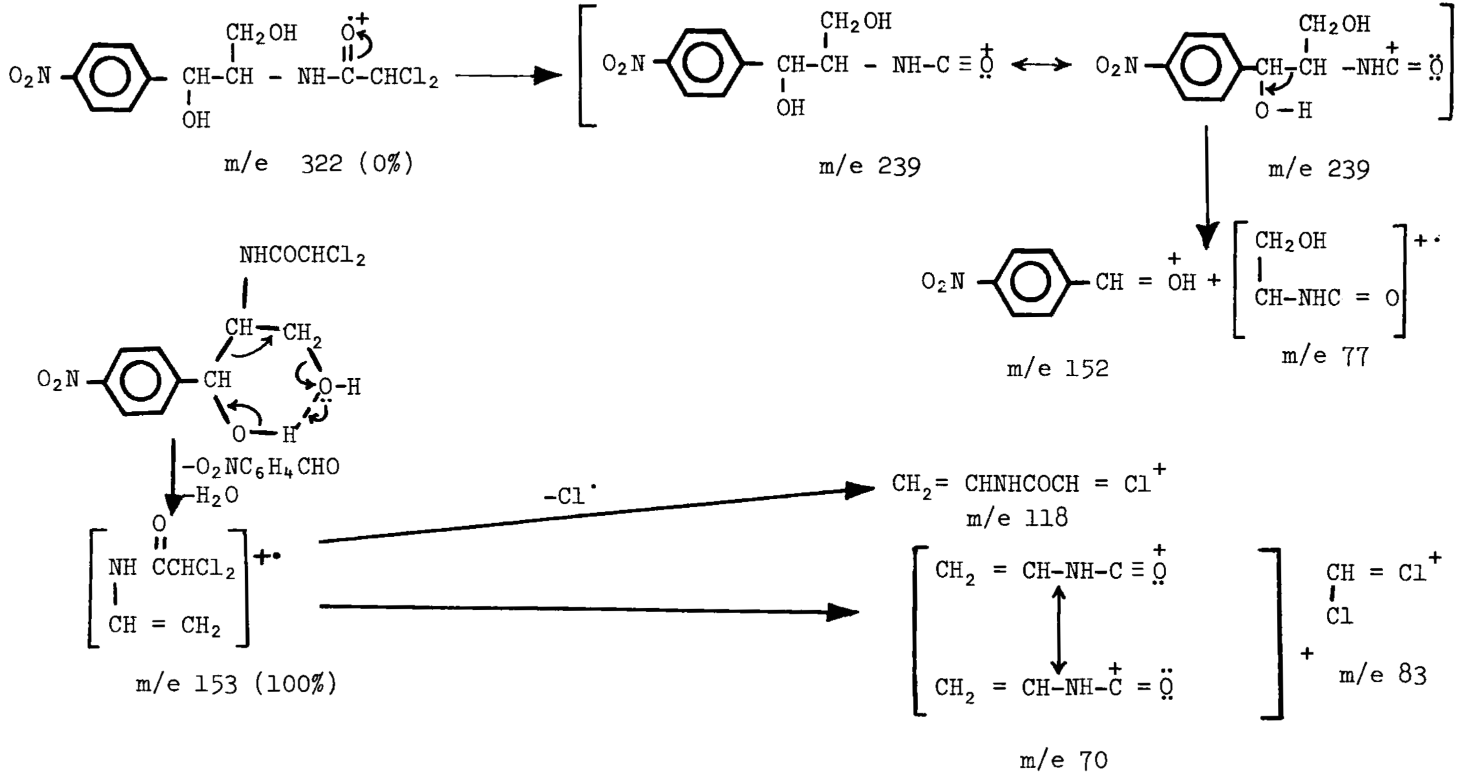


Figure 8. Mass spectrum of chloramphenicol (CI).



Scheme 1. Mechanism of chloramphenicol fragmentation.

Scheme I (continued)

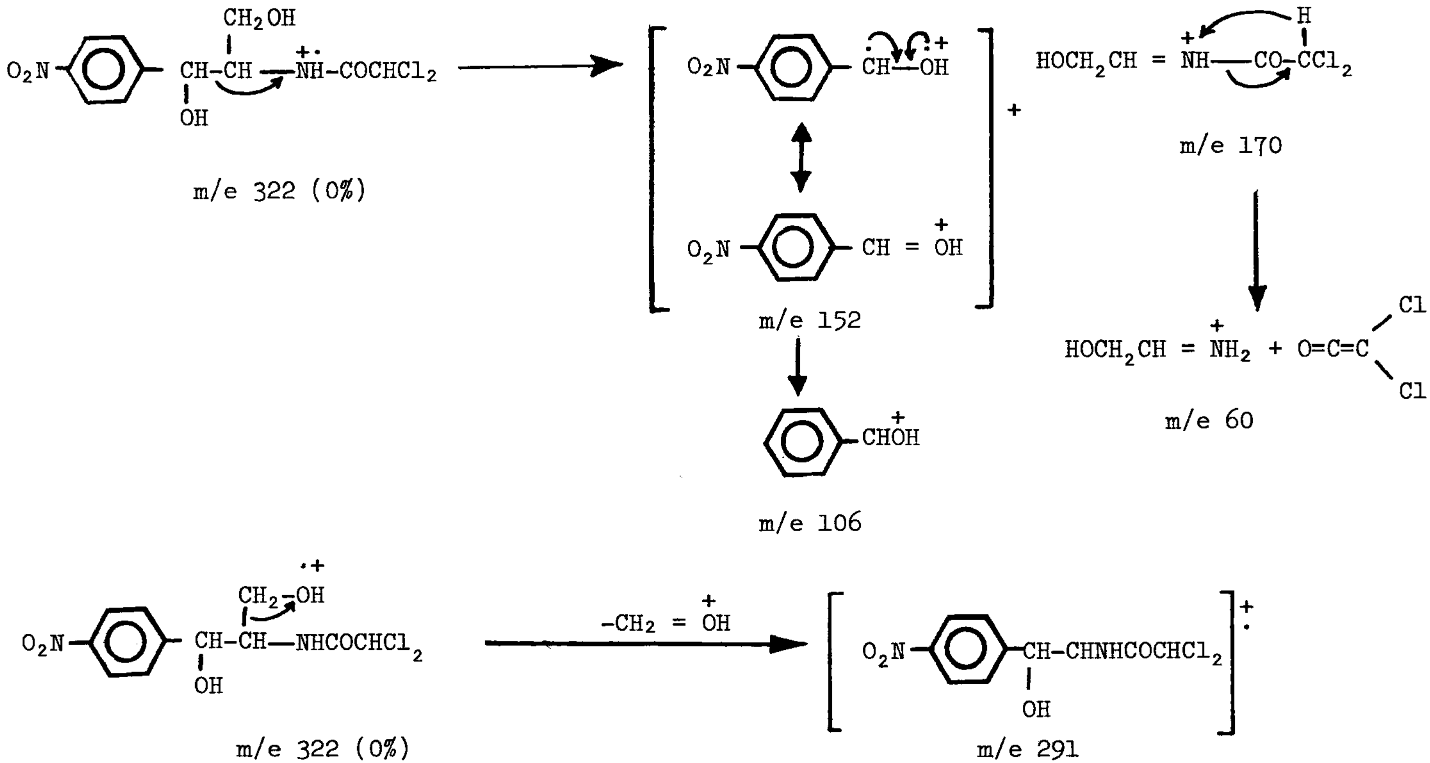


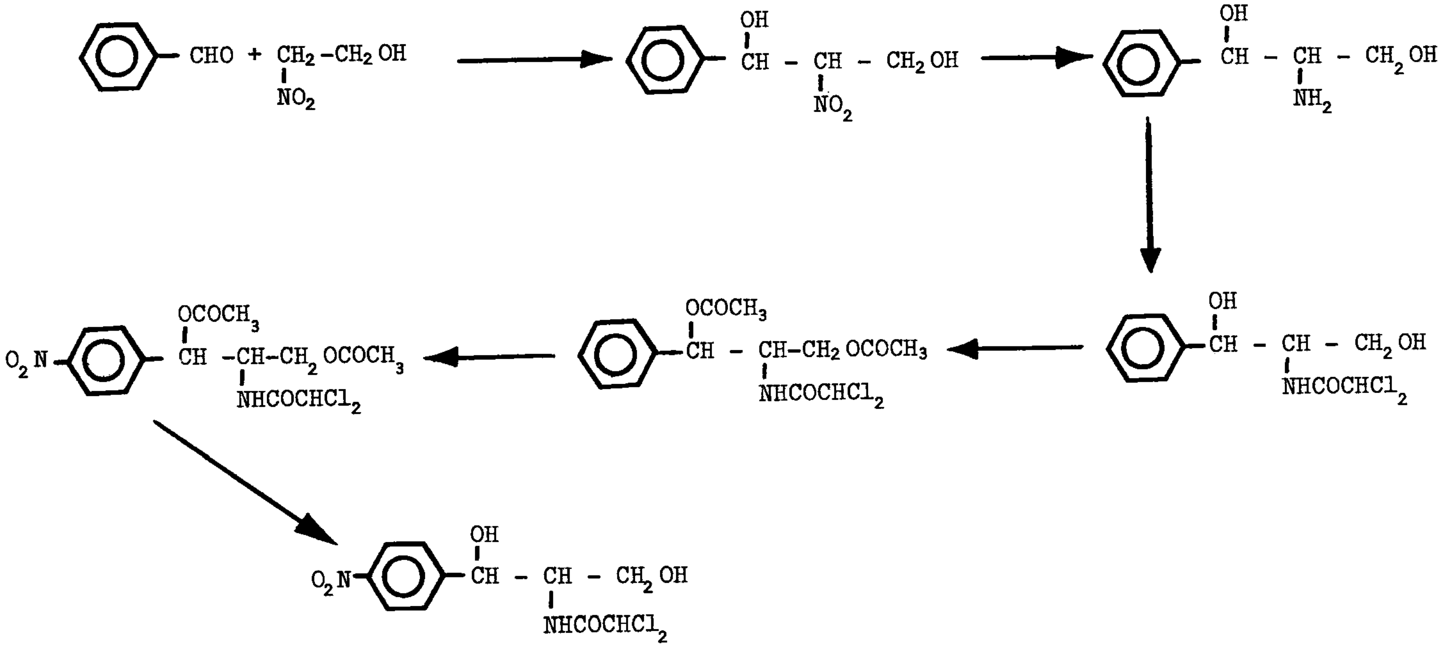
Table 1. Mass Spectral Assignments of Chloramphenicol Using CI with Methane as Reagent Gas.

m/e	Species
363	$[M + C_3H_5]^+$
351	$[M + C_2H_5]^+$
323	MH^+
305	$[MH - H_2O]^+$
287	$[MH - HCl]^+$
275	$[MH - (H_2O + CH_2 = O)]^+$

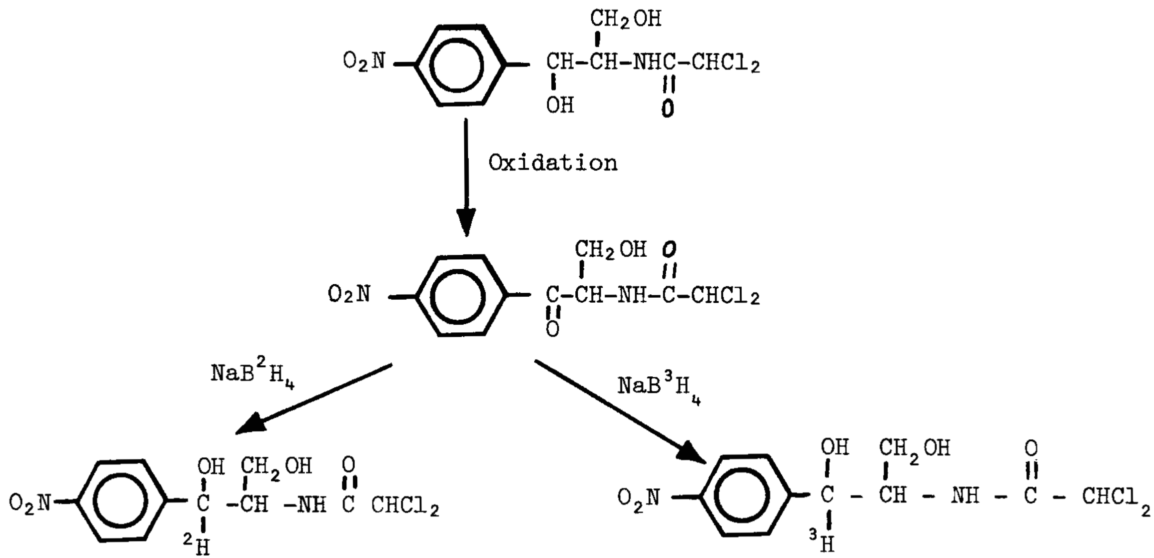
3. Synthesis

a. The antibiotic was initially isolated from cultures of various *Streptomyces* strains. The structure simplicity of chloramphenicol made it amenable to preparation by total synthesis both in the laboratory and on commercial scale. One method of synthesis involves a base-catalysed condensation of benzaldehyde with nitroethanol to afford the aldol product as a mixture of stereoisomers (Scheme 2). Catalytic reduction gives an aminodiol whose *threo*-isomer is separated and resolved into the optical isomers. The (-) isomer is treated with dichloroacetyl chloride followed by treatment with base to remove the O-acylated products to afford the amide. The hydroxyl groups are then protected by means of acetic anhydride. The product is nitrated to produce the p-nitroderivative. Removal of the protecting groups is achieved by treatment with a base to give chloramphenicol (9).

b. ^3H-Chloramphenicol and its *erythro*-diastereomer with high specific activities were prepared by oxidation of the nonlabeled antibiotic to its oxo derivative (Scheme 3), which upon reduction with ^3H-sodium borohydride was converted to the corresponding diastereomers. The diastereomers were separated by HPLC. ^2H-Chloramphenicol diastereomers can be synthesised similarly using ^2H-sodium borohydride (10).



Scheme 2. Synthesis of chloramphenicol.



Scheme 3. Synthesis of labeled chloramphenicols.

4. Methods of Analysis

4.1 Identification Tests

a) Dissolve about 10 mg in 1 ml of alcohol (50%), add 3 ml of a 1% of calcium chloride and 50 mg of zinc powder, heat on a water bath for 10 minutes, cool and filter; to the filtrate add 100 mg of anhydrous sodium acetate and 2 drops of benzoyl chloride, shake for 1 minute and then add 0.5 ml of ferric chloride solution and 3 ml of dilute hydrochloric acid and mix; a reddish-violet or purple color is produced. No such color is produced when the test is repeated without zinc powder (11).

b) To 5 ml of 0.1% solution add a few drops of silver nitrate solution, no precipitate is produced. Heat about 50 mg with 2 ml of alcoholic potassium hydroxide solution on a water bath for 15 minutes, add a small quantity of decolorizing charcoal, shake, filter and to the filtrate add silver nitrate solution; a white precipitate is produced which is insoluble in nitric acid but soluble after washing with water, in dilute ammonia solution (11).

c) Dissolve about 10 mg in 2 ml of alcohol (50%) add 4.5 ml of dilute sulfuric acid and about 50 mg zinc powder, allow to stand for 10 minutes and decant the supernatant liquid; cool the supernatant liquid in ice; add 0.5 ml sodium nitrite solution, allow to stand for 2 minutes and then add 1 gm of urea followed by 1 ml of 2-naphthol solution and 2 ml of sodium hydroxide solution; a red color is produced (11).

d) The nitro group is reduced to an amino group by zinc-HCl and the amine is caused to react with dimethylaminobenzaldehyde, the resulting Schiff base gives a colored salt in acid medium. The method can be applied as a spot test. Chloramphenicol in ointments is extracted into 96% ethanol. Riboflavine does not interfere in the detection of chloramphencol (12).

e) An orange-red color is produced and ammonia is evolved when chloramphenicol is heated with 50% NaOH solution (3).

f) Chloramphenicol gives a positive reaction to Fujiwara's Test (3) as follows: a little solid or drop of a test solution is added to a mixture of pyridine (1 ml) and 20% NaOH solution (2 ml). The mixture is heated on a boiling water bath for 3-5 minutes with vigorous periodic shaking. A control test must be carried out. A red color appears in the pyridine layer.

g) Ammonium molybdate test for micro quantities gives faint blue color (3).

4.2 Quantitative Analysis

4.2.1 Biological Methods

4.2.1.1 Microbiological Methods

Chloramphenicol bioassay have been reported by Bannatyne and Cheung (13). The authors described an accurate plate diffusion bioassay for the drug, in which the fast-replicating *Beneckea natriegens* and 1.5% salt agar are used. Zone of inhibition were well defined after 3 hours and the limit of sensitivity of the method was around 2 μg/ml.

Fabiansson and Rutegaerd (14) have reviewed the biological methods in current use for the detection of antibiotic residues in slaughter animals and reported a modified method in which the conditions for the control were standardized. The standardized conditions include the use of a sporulating organism, *Bacillus subtilis*, an inoculum size of 0.5×10^5 spores/ml medium, add 5 ml of medium (pH 6.0) per plate. A preincubation diffusion time of

1 hour at room temperature is recommended before incubation.

Horng and Ko (15) have reported systematic analysis of antibiotics via agar gel electrophoresis and antimicrobial spectrum of methodology. Antibiotics are detected in food and drugs, by a method which employed both agar gel electrophoresis and antimicrobial spectrum.

Horng _et al_ (16) have also reported systematic analysis of antibiotics via agar gel electrophoresis and antimicrobial spectrum-candidacy for detecting residual antibiotics in foods. The method is a modification of the method of Horng and Ko (15) to increase its resolution power and sensitivity. Modification included the amount of agar gel and the height of the test organism strip and the width of sample slit and enabled the analysis of antibiotics at food residue concentrations (1 µg/ml).

4.2.1.2 Enzymatic Methods

Smith and Smith (17) have reported an improved enzymic assay of chloramphenicol. R-factor-incoded chloramphenicol acetyl transeferase, from an _Escherichia coli_ mutant that was highly resistant to chloramphenicol was partly purified and used for the assay. Only antibiotically active chloramphenicol is attached by the enzyme. Crystalline chloramphenicol is dried to constant weight at 60° and dissolved in serum, and 50-µl portions (as standards) are are stored at -70°. Serum samples and standards are heated at 60° for 15 minutes before addition of 10 µl to 50 µl of [^{14}C] acetylcoenzyme A

solution (pH 7.8) and 25 µl of enzyme source (0.1 spectrophotometric unit). After incubation at 37° for 60 minutes, the diacetylated product is selectively absorbed on micropore filters and then assayed by scintillation counting.

Robison et al (18) have developed a simplified radio-enzymatic assay for chloramphenicol, by elminating the need for cumbersone extraction procedure. After the acetylation of chloramphenicol with ^{14}C-labeled acetyl CoA in the presence of chloramphenicol acetyltransferase the reaction mixture was added to a toluene-based scintillation fluid. Since ^{14}C-acetylated chloramphenicol is more soluble than ^{14}C-labeled acetyl CoA in toluene, the radioactive product could be counted directly.

Detection and quantitation of chloramphenicol by competitive enzyme-linked immunoassay was reported by Campbell et al (19). The assay for the drug in meat involves competitive inhibition, by free chloramphenicol in the sample, of the binding of specific rabbit antibody to solid-phase-bound chloramphenicol. The antibody not displaced was measured by using a commercially available enzyme-linked anti-rabbit 1 gm prepration and added substrate. Enzyme activity, measured spectrophotometrically, was inversely proportional to the concentration of chloramphenicol in the sample.

4.2.2 Chemical Methods

4.2.2.1 Titrimetric Methods

Navik and Polyakova (20) have reported the analysis of some multi-

component water-alcohol mixtures where aqueous ethanol solution of chloramphenicol was determined by a titrimetric method.

El-Sebai et al (21) have described new internal indicators for the determination of primary aromatic amines (chloramphenicol yields an amino group on reduction with zinc dust and hydrochloric acid). Various diazo- compounds were synthesized and tested as an indicators in the titration of such amines with $NaNO_2$ solution.

Talegaonkar et al (22) have described an alkalimetric determination of chloramphenicol in dimethylformamide. A solution of the drug in dimethylformamide is titrated with sodium methoxide solution in benzene-methanol 4:1, with 2 drops 1% 2-nitroaniline solution in benzene as indicator, the color change is from yellow to red. The reaction involved in the titration is replacement of one chlorine atom in the drug by a methoxy group.

Koka (23) has described an iodimetric method for the determination of chloramphenicol in some medicinal mixtures. The sample is boiled with sodium hydroxide solution, cooled, diluted, treated with 0.1N - iodine, set aside for 10 to 15 minutes in the dark, and then treated with potassium iodide solution and dilute sulphuric acid, and the liberated iodine is titrated with sodium thiosulphate solution using starch as indicator.

Koka and Koltun (24) have reported another iodimetric method for the determination of chloramphenicol in

some other medicinal forms.

4.2.2.2 Polarographic Methods

Chloramphenicol [in milk] was determined by Fossdal and Jacobsen (25) polarographically. The electron-reduction of the drug was studied by polarography of 0.5 mM solution of various electrolytes and of 1.7 mM to μM solution in 0.5M-acetate buffer at pH 4.7 and by cyclic voltammetry, chronopotentiometry and coulometry; the reactions involved are discussed. A well-defined polarographic wave was obtained which even in the presence of 50% of milk is analytically useful over the range of 0.3 to 60 μg of antibiotic per ml.

Chloramphenicol and it hydrolysis product, 2 amino-1-(p-nitrophenyl)-1,3-propanediol were determined polarographically in pharmaceutical formulations (26), after separation by thin-layer chromatography on silica gel GF_{254} using a 4:1:1 n-butanol-acetic acid-water or 2:2:4 acetone-benzene-petroleum ether solvent mixtures. The separation of the above two compounds by high-pressure liquid chromatography (Perkin-Elmer reverse phase C_{18} column with 45:55:1 methanol-water-acetic acid or 30:70:1 isopropanol-water-acetic acid) made possible simultaneous determination of the above two compounds.

Polak _et al_ (27) have determined chloramphenicol in body fluids by differential pulse polarography. Reduction of the drug at a dropping-mercury electrode at -0.4 V (vs the s.c.e) at pH 4.5 (Britton Robinson buffer) is used for the determination

of the drug in such samples after precipitation of proteins with acetonitrile or methanol. The coefficient of variation was 4% for determination of 15 µg/ml of chloramphenicol in serum. Conventional d.c. plarography is suitable for determining the drug only in urine.

4.2.2.3 Colorimetric Methods

Several colorimetric methods for the determination of chloramphenicol have been reported in the literature.

Plourde and Braun (28) have described a colorimetric procedure for the determination of the drug in tablets and capsules. For tablets, powder the material and dissolve a sample containing 20 mg of chloramphenicol in 50 ml of 1,2-dichloroethane at 60°, cool and dilute to 100 ml with dichloroethane. Filter the solution and reject the first few mls. For soft capsules, section longitudinaly, and dissolve in dichloroethane (150 ml) at 60°, cool and dilute to 250 ml with dichloroethane. Filter, reject the first few mls. and dilute an aliquot containing 20 mg of chloramphenicol to 100 ml with dichloroethane. To this dilute solution (4.5 ml) add dichloroethane (6.5 ml) and 'piperidine-8-hydroxyquinoline vanadate' reagent (4 ml) and leave for 30 minutes at room temperature. Extract excess reagent with M-NaOH (10 ml) for 30 seconds, set aside for 30 seconds and filter the organic phase over Na_2SO_4 into 1 ml of 5% dichloroacetic acid solution in acetic acid then measure the extinction of the resulting blue solution at 625 nm within 2.5 hours.

A colorimetric method using p-dimethylaminobenzaldehyde is described (29) for the determination of chloramphenicol and other compounds. In an acid medium p-dimethylaminobenzaldehyde produced reaction products varying in color from yellow to deep red. The reaction products showed maximum absorption spectrum peaks between 425 and 450 nm.

Ivakhnenko _et al_ (30) described a procedure for absorbtiometric determination of chloramphenicol. Dilute the reduction product of 0.5 gm of chloramphenicol to 100 ml. To 1 ml of the solution add 5 ml of N-HCl, 2 ml of 0.01M - $NaNO_2$ and, after 4 to 5 minutes, 5 ml of 0.3% solution of a diaminoacridine reagent (ethacridine lactate or proflavine); after a further 2 minutes dilute the solution to 50 ml and measure the extinction of the resulting diazo-compound at 508 nm for the first reagent or at 584 nm for the other against water.

The determination of chloramphenicol in pharmaceuticals (suppositories and coated tablets) have been reported by Cieszynski _et al_ (31) using colorimetry. The product of the reduction of chloramphenicol reacts with guaiacol in alkaline medium (pH 9.6) to form a blue complex, which is stable for up to 5 hours, with measurement of the extinction, at 610 nm, 30 minutes after the solutions are mixed.

Przyborowski (32) described a method for the determination of the drug and its palmitate in pharmaceuticals. The method involves the hydrolysis of chloramphenicol by NaOH in a medium containing hydroxylammonium chloride, and reaction of the result-

ing 2,2-dichloroacetohydroxamic acid with Fe^{3+}. The coloured complex produced is determined by spectrophotometry at 505 nm. A modified method for the determination of chloramphenicol palmitate and the contents of chloramphenicol and chloramphenicol palmitate in several preparation is also given.

Krezk and Lechniak (33) have reported the application of copper (II) to the colorimetric determination of chloramphenicol in ointments. The method is based on the formation of a complex after mixing methanolic solutions of chloramphenicol, copper (II) and methanol; the precipitate of $Cu(OH)_2$ is filtered and the absorbance of the filtrate is measured at 550 nm. The composition of the complex corresponds to copper: chloramphenicol molar ratio = 1:2.

Catechol and iodine have recently been used for the spectrophotometric determination of aromatic amines (34). The method, involves mixing 15 ml of potassium acid phthalate buffer solution (pH 3.1), 1 ml of aqueous 0.1% catechol, 1 ml of 0.01N-iodine and 1.5 ml of the amine solution, dilution of the mixture to 25 ml with water, and, after 5 to 30 minutes (depending on the amine), spectrophotometry at 500 to 520 nm (vs a reagent blank). A modification of this procedure is described for compounds that yield primary arylamino-groups on reduction (chloramphenicol).

Yang (35) has described a simultaneous determination of chloramphenicol and its metabolite D-threo-2-amino-1-(4-nitrophenyl)propane-1,3-diol in injections. A 10 ml sample

is diluted to 25 ml with anhydrous ethanol and a portion is sampled for spectropolarimetric determination of the specific optical rotation at 418 and 589 nm for the drug, and its metabolites, respectively.

Divakar *et al* (36) have used brucine and sodium metaperiodate for the colorimetric estimation of chloramphenicol. A 10-ml portion of the test solution was boiled under reflux for 45 minutes with 10 ml of 2M-HCl, and the excess of HCl was removed *in vaccu*. The residue was dissolved in 20 ml of warm water, and the solution was diluted to 50 ml with water. This solution in test tubes was then treated with 3 ml of 5 mM-brucine, 1.5 ml of 5 mM-$NaIO_4$ and 2 ml of 2.3 M-H_2SO_4 and the solution was diluted to 10 ml with water.

The test tubes were shaken and heated on a boiling-water bath for 15 minutes. After cooling the contents of each tube were diluted to 25 ml with water and the absorbance of the solution was measured at 500 nm.

4.2.2.4 Ultraviolet Spectrophotometric Methods

Chloramphenicol was determined in pharmaceutical preparations containing boric acid, glycerin, sodium chloride, zinc sulphate, belladonna extract, streptocid, glucose, or resorcinol by ultraviolet spectrophotometric analysis at 278 or 315 nm. The drug was determined with a relative error of ± 3.46 (37).

Buryak (38) reported the analysis of multicomponent medicines with the aid of a computer. The sample is

boiled for 5 minutes with 95% ethanol, and the extract is filtered and diluted as necessary with ethanol. The absorbance is measured at various wavelengths between 226 and 330 nm vs ethanol. The concentration of individual components are calculated from a matrix of equations relating the total absorbance to the sum of the partial absorbances of the components. The method is suitable for lanolin-based ointments containing chloramphenicol.

4.2.2.5 Infrared Spectrophotometric Methods

Infrared spectroscopy had been used for the determination of the antibiotic stability (39). The effect of external factors such as heat, acidity and hydrolysis on crystalline antibiotics was examined spectroscopically. Chloramphenicol resisted dry heat at 80° for 24 hrs, but was unstable at 100° for 2 hrs.

Namigohar et al (40) reported an infrared spectrophotometric determination of chloramphenicol. The drug was extracted from capsules, creams and eye drops, with ethanol or ethyl acetate-chloroform and chloramphenicol palmitate was extracted with chloroform. Chloramphenicol and chloramphenicol palmitate were then determined by infrared spectrophotometry, in KBr and in chloroform, respectively.

4.2.2.6 Proton Magnetic Resonance Spectrometric Methods

Chloramphenicol in pharmaceutical preparation has been determined using proton magnetic resonance spectrometry (41). The drug was dissolved in dimethyl sulfoxide containing

maleic acid as internal standard. The n.m.r spectrum of the solution was recorded, and the peaks for the aromatic protons of chloramphenicol at 7.6 and 7.5 ppm and the vinylic protons of maleic acid at 6.25 ppm were integrated; the amount of chloramphenicol was calculated from the integration ratio and the known amount of maleic acid. For ten samples containing 100 to 150 mg of pure chloramphenicol, the average recovery was 100.22% (standard deviation 1.37%). The method has been applied to the analysis of commercial capsules and oral suspension of chloramphenicol palmitate; it is rapid and simple and can also be used to check the purity of the drug.

4.2.2.7 Mass Spectrometric Methods

Mass spectrometric methods have been described for the analysis of chloramphenicol in aqueous solutions (4) and in animal tissues and body fluids (5,6,52).

4.2.3 Chromatographic Methods

A multitude of thin layer, paper, column, gas and liquid chromatographic methods have been developed for the detection and determination of chloramphenicol in pharmaceutical formulations, biological fluids and animal tissues.

4.2.3.1 Thin Layer, Paper and Column Chromatography

Various thin layer, paper and column chromatographic methods used for the analysis of chloramphenicol are outlined in Table 2.

4.2.3.2 Gas Chromatographic Methods

Gas chromatographic methods have been used for the determination of chloramphenicol in dosage forms and biological fluids and tissues. Table 3 summarises some of these methods.

4.2.3.3 High Performance Liquid Chromatography (HPLC)

HPLC has been extensively used for the determination of chloramphenicol in pharmaceutical formulations and biological fluids as well as for the detection and determination of the drug residues in animal tissues. Some of these methods are outlined in Table 4.

Table 2. Thin Layer, Paper and Column Chromatographic Methods for the Analysis of Chloramphenicol.

Support	Solvent System	Detection	Ref.
Silanized silica gel (reverse phase)	Mixture of solvents e.g. Dioxane, acetone, isopropyl alcohol, methanol, tetrahydrofuran or ethyl methyl ketone with citrate-phosphate buffer (pH 3, 5 or 7).	-	42
Silica gel	$CHCl_3$-methanol-2.5% aq. NH_3 (60:6:1)	Spray with $SnCl_2$ solution, heat to 110° for 7 min and spray with 4-dimethylaminobenzaldehyde.	43
Silutol UV 254 Sheet	Ethyl ether.	Fluorescence quenching	44
Silica gel	CH_2Cl_2-ethyl acetate (1:4).	15% $SnCl_2$ in aq. HCl, then UV.	46
High performance TLC contact spotter	Chloroform-heptane-methanol (4:2:1)	UV at 280 nm.	46
Silica gel GF_{254}	n-Butanol-acetic acid-water (4:1:1) or Acetone-benzene, petroleum ether (2:2:4)		26

Table 2 (Continued)

Support	Solvent System	Detection	Ref.
Whatman No. 1 paper	2.5% Acetic acid in butanol water (22:3).	4-Dimethylaminobenzaldehyde.	47
17.5 cm x 2 cm column of neutral alumina	Ethanol-ethylacetate-aq. NH_3 (50:50:1)	Biological assay	48

Table 3. GC Methods for the Analysis of Chloramphenicol

Packing Material	Column Temperature (°C)	Detector	Ref.
3% Dexil 300 on Supelcoport AW-DMCS (100-120 mesh).	250	ECD	49
1.5% OV-17 on Supasorb M AM-DMCS	260	FID	50
3% DC-200 on Chromosorb W DMCS (80 to 100 mesh).	190	ECD	51
OV-1	Temperature programming for 190 to 220 at 6° min^{-1}.	MS	52
3% OV-1 on Gas-Chromo. Q (100 to 120 mesh).	220	ECD	53

Table 4. HPLC Methods for the Determination of Chloramphenicol

Column	Mobile Phase	Detector	Ref.
Micropak CN	Hexane-CH_2Cl_2-methanol.	UV at 254 nm	54
A reverse phase column	Acidified ethanol-water	UV	55
DVB-MCL-O	Methanol-ammonia.	UV	56
RP-2 (10 µm)	0.01M K_2HPO_4 -methanol (29:21).	UV	57
Partisil-10 ODS	50 mM KH_2PO_4 (pH 4.5).	UV at 254 nm	58
µ Bondapak phenyl	0.05M H_3PO_4 -acetonitrile (3:1)		59
µ Bondapak C_{18}	20% Acetonitrile in 0.05M Na acetate buffer (pH 5.3).	UV at 278 nm	60
Sep-Pak C_{18}	Ethyl ether and ethanol.	UV	61
Nucleosil C_{18} (5 µm).	Water-methanol (7:3).	UV at 278 and 350 nm	62
Hypersil H_5 ODS	Water-acetonitrile-acetate buffer (80:20:1).	UV at 276 nm	63
µ Bondapak C_{18}	Aqueous methanol.	UV	64

Table 4. (Continued)

Column	Mobile Phase	Detector	Ref.
μ Bondapak C_{18}	Acetonitrile-phosphate buffer (1:3)	UV at 278	52
RP-18	35 to 40% aq. methanol containing 100 mg/L of K_2HPO_4.	UV	45
Radial-PAK C_{18} with RCSS Guard-PAK precolumn.	Methanol-0.75% acetic acid (3:7) adjusted to pH 5.5 with triethylamine.	UV at 280 nm	65
C_{18} Varian Micro-pak MC H (10 μm)	Phosphate buffer (pH 3.25)-methanol-acetonitrile (27:9:4).	UV at 278 nm	66
Perkin-Elmer Reverse Phase C_{18} column.	Methanol-water-acetic acid (45:55:1) or Isopropyl alcohol-water-acetic acid (30:70:1).	Polarographic	26
μ Bondapak C_{18}	Water-methanol-acetic acid	UV at 280	67

5. Pharmacokinetics

5.1 Absorption and Distribution

Oral doses of 1 gm chloramphenicol produce peak levels of 10-20 µg/ml at 2 to 4 hr. (68, 69). The serum level peak following oral administration is approximately the same as that obtained following IV administration, although peak levels are reached slower by the former route. Yogev et al (70) conducted a study in which 39 children with H influensae meningitis were treated for 5 days with oral chloramphenicol. All patients responded well to therapy and no relaspse developed. The route of administration had little impact on the pediatric patients treated and the serum levels after oral administration was equal to or greater than IV formulations. Chloramphenicol palmitate administered to children between the age of 2 months and 14 years orally every 6 hr in doses of 60-70 mg/kg/day resulted in a serum concentration (at steady state) ranging from 15.5 to 29.0 µg/ml with a mean of 20.2 µg/ml after 90 min from administration (71). Chloramphenicol succinate administered every 6 hr by IV route to 18 children between the age of 2 months and 14 years in doses of 60 to 109 mg/kg/day resulted in a serum concentration (at steady state) ranging from 12.5 to 43.1 µg/ml after 90 min. from administration (71). Lower blood levels are produced by 1M chloramphenicol sodium succinate than by identical IV doses (72) and about 50% the serum level obtained after identical doses given by oral route (5-6 gm/ml) (69). Oral administration of chloramphenicol resulted in about 75-90% absorption with peak levels occuring 0.5 to 2 hr following administration (73, 74). The palmitate ester, however, must be hydrolysed before absorption. Hydrolysis may be inadequate in newborns, infants and children and absorption delayed and unreliable (75,76). Chloramphenicol base administered orally produces peak serum levels equivalent to or higher than IV administration (77, 78). Peak levels of 10-13 µg/ml are obtained in about 2 hr after the administration of 1 gm oral dose, and sustained administration every 6 hr provides cumulative effect with somewhat higher peak levels (68,79). Chloramphenicol sodium succinate, using 1 gm IV dose, produces similar blood peak levels

only occuring immediately. Blood levels of the same order are obtained in children with an equivalent single oral or IV dose (79). In newborn infants 2,4, or 12 hr after chloramphenicol succinate administration (12.5 mg/kg, IV) serum chloramphenicol concentrations were < 10 mg/L at each time studied. After loading dose of 20 mg drug/kg the mean serum drug concentrations were higher in infants $\leq$ 2 day old than in infants $\geq$ 3 days old (80).

The effect of dosing methods on chloramphenicol absorption was studied (81) using young yellow tails. The absorption is found to be greater in prportion to the increase in the drug content in the feed. Of several diets tested with varying concentration of the drug, a diet containing 50% of the drug and 0.2% of a binder produced the highest drug level in the fish.

The absorption of chloramphenicol was studied (82) in 6 normal volunteers by various routes of administration. Peak serum levels were maximum with the oral route of administration. With the lM route the average peak level was only 70% of that of the oral route. The absorption of the drug was minimum and variable after rectal administration. Animal studies were conducted for improving the rectal absorption of chloramphenicol (83). The drug rectal absorption was found to be strongly species-dependent. In rabbits the drug absorption was greatly increased after rectal administration of chloramphenicol - erythromycin mixture and chloramphenicol - oleandomycin mixture, compared to absorption after administration of other chloramphenicol mixture of the drug esters. The increase in absorption was not observed in people, rats and guinea pigs. Chloramphenicol esters e.g. chloramphenicol palmitate must be hydrolysed by pancreatic lipases in the duodenum before absorption takes place. Accordingly the rate of hydrolysis of the palmitate is a major factor in determining the ultimate blood levels achieved. That the absorption of the palmitate is slower has been shown by Weiss et al (75) in their studies in newborn infants. In older children, it is also reported (76), up to 50% of an administered dose of palmitate may be lost in the feces. The dose of the palmitate must be higher than with the crystalline chloramphenicol base (100-200 mg/kg/day). This is in agreement with the results of earlier studies (84, 85).

The slow hydrolysis resulting in lower blood levels reported by some investigators is due to the separate polymorphic states in which the palmitate can exist. One crystalline form is substantially more hydrolysed than the other and the blood levels observed are directly related to the proportion of that form which is present in the preparation (86). Subsequent to these reports, however, Park-Davis Company has indicated that the less hydrolysable polymorph of the palmitate has been removed from the preparation and the absorption of chloramphenicol from the palmitate is now complete and reliable, producing blood levels equivalent to IV administration (78). A prospective, randomized evaluation of oral chloramphenicol administration for completion of therapy of H influenzae type b meningitis is conducted (87) in 44 children: 21 patients received the drug orally after the second day of therapy, the remainder continued to receive the drug via IV route. There was equivalent bioavailability of chloramphenicol. In 43 patients the resolution of clinical manifestations and CSF abnormalities of meningitis was equivalent with both routes. These findings indicate that higher than normal doses of chloramphenicol palmitate are not necessary. A 6 hourly devided dosage of 100 mg/kd/day will produce blood levels equivalent to those obtained by IV or oral administration of the base. Adequate CSF level will be maintained since concentrations of chloramphenicol in the CSF reach levels as high as 50% of that obtained in the blood and are well above the MIC for H influenzae. The absorption of chloramphenicol after oral administration in severely malnourished children was found to be erratic. This route should be avoided in such patients (88).

The bioavailability of the oral chloramphenicol palmitate salt was investigated (71) and compared to that of IV succinate salt in 18 children, age 2 months to 14 years. The bioavailability of the oral dose was found to be greater than the IV preparation. The relative bioavailability of the succinate compared to the palmitate was 70%. This can be explained by the prominent interpatient variation in the extent of hydrolysis of the IV succinate salt to the biologically active chloramphenicol when compared to the oral salt form. In this study a mean of 36% of the administered IV dose was excreted

unchanged in urine. This value probably accounts for the 30% reduction in bioavailability of the IV dosage form when compared to the oral form which is more completely hydrolysed to the active form in the GIT.

Simultaneous administration of vitamins with the antibiotic has been found to reduce chloramphenicol blood levels (89).

Chloramphenicol is widely distributed in the body with therapeutic levels occuring in most body cavities, the eye and CSF. Chloramphenicol is 60 to 80% protein bound (73). Other studies, however, suggest that it is about 36% protein bound (90).

There is no literature report on complete pharmacokinetic profile of chloramphenicol in the cerebrospinal fluid. Although many studies have documented the achievement of therapeutic levels in the CSF, only one has measured sequential CSF levels over an entire dosing period (91). A male adult patient with H influenzae meningitis received oral chloramphenicol 12.5 mg/kg every 6 hr. Serum and CSF levels were measured after the 7th dose to ensure steady state kinetics. The CSF levels were 5.4 µg/ml at 0 hr, 5.7 at 30 min, 6.3 at 1 hr, 7.4 at 2 hr, 7.2 at 3 hr and 7.9 at 6 hr. The mean CSF/serum ratio was 36%. Results of isolated experiments is included in Table 5.

The reported results emphasise the considerable individual variations, probably due to varying degrees of miningeal inflammation, the drug appears to produce very good CSF levels when sufficient doses are administered. With IV administration the CSF/serum ratios range from 22.5-99%, with steady state CSF levels from 4-23.3 µg/ml. Oral dosages produce similar and possibly higher CSF levels with CSF/serum ratio from 20-60% and CSF levels of 4-32 µg/ml.

A comparative study (100) of the steady-state CSF levels after IV or oral doses of 100 µg/kg/day of chloramphenicol in 14 patients showed that serum levels after IV administration occured at 45 min. With mean corresponding to CSF levels of 4.2 µg/ml. Peak serum levels resulting from oral dosing occured

Table 5. Summary of CSF Levels Reported in Isolated Determinations *

Dose (mg/kg/D)	Dosage form	CSF* level (μg/ml)	Serum level (μg/ml)	CSF/Serum ratio (%)	Ref.
7 - 15	Oral	0	4 - 32	0	92
7 - 15		4	20- 64	6 - 20	
9 - 45		8	32- 40	20-40	
50		32	128	25	
28 - 66	Rectal	0	2 - 3.6	0	93
50 - 68	Oral (single)	4 - 10.3	12.2 - 34	25-42	
18 - 26	Oral (multiple)	0 - 16.7	1 - 42	0 - 39	
Unknown	IM (single)	0 - 4.2	5.1 - 23	0 - 28	
18 - 72	IM	3.2 - 9.9	24.8 - 74.4	6 - 27	
66	Oral	2 - 8.9	3 - 21.8	50	94
50	IV	14	25	56	95
25 - 35	IV	mean 23.3 +/- 7.7	14.1 - 54.4	50 - 87	96

*Through Drugdex, Microfiche System, Micromedex, Inc., Englewood, Colorado, U.S.A.

Table 5 (Continued) ...

Dosge (mg/kg/D)	Dosage form	CSF level (µg/ml)	Serum level (µg/ml)	CSF/Serum ratio (%)	Ref.
60	IV	4(peak after 3 hr)	16.5(peak after 30 min)	22.5	97
75		11.5(peak after 3 hr) (Ventricular fluid)	20(peak after 30 min)	57.5	
12.5 - 25 (neonates) 40 - 100 (Older children)	IV	4 - 18	8 - 40	45 - 99	98
100	IV	5.5 - 13	10 - 29.5	23 - 85	99
100	IV	mean 4.2 (1 - 7.5)	mean peak 15 at 45 min.	mean 65	100
100	Oral	mean 6.6 (1.5 - 11.5)	mean peak 81.5 at 2-3 hr.	mean 60	

at 2-3 hr with CSF levels of 6.6 µg/ml.

Four premature infants under 5,500 gm were treated with parenteral chloramphenicol for central nervous system infection due to organisms resistant to the penicillins. Serum, cerebrospinal fluid (CSF) and ventricular fluid concentration of the drug were measured frequently during therapy and were used to maintain drug dosages in the safe and therapeutic range. Concentration of the drug in the lumbar CSF and ventricular fluid had a mean of 23.3 µg/ml, consistently greater than 45% of peak serum levels (96). The data show that chloramphenicol enters the CSF in both ventricular and lumbar regions in therapeutic concentrations when administered I.V. The distribution of ^{14}C-labeled D(-)-threo-chloramphenicol was studied (101) in newborn pigs by whole-body autoradiography. The amount of radioactivity in the lung, liver, adrenal cortex, kidney, myocardium, pancreas, thyroid, spleen and skeletal muscles was higher than that in the blood short time after the injection and remained higher upto 8 hr. After 4 and 8 hr the brain concentration of ^{14}C was also higher than that of the blood. In the bone marrow, however, the concentration did not reach that of the blood during the whole experiment. In the organs $> 90\%$ of the radioactivity was represented by unchanged chloramphenicol; the excretory organs, thyroids and adrenals being exceptions. In the study (81) using cultures yellowtail, Seriola quinqueradiala, the distribution of chloramphenicol follows the order: liver > muscle > blood. The volume of distribution for chloramphenicol was reported to be about 40 litres (74).

5.2 Excretion

Chloramphenicol is reported to be 5 - 15% excreted unchanged (73,102) with reported renal clearance of 13-36 ml/min (73). Renal levels may be inadequate to treat urinary tract infections especially in the presence of moderately to severely impaired renal function (103). Some normal patients and patients with impaired renal function exhibit impaired free drug elinination (104). The recovery of free drug from the urine is directly proportional to creatinine clearance. With creatinine clearances of less than

20 ml/min, less than 1% of the administered doses that are recovered in the inert inactive form. With creatinine clearances of < 40 ml/min, urinary concentrations of the drug are generally not high enough to treat susceptible organisms (105). The steady state kinetics of the oral palmitate versus the IV succinate salt was studied (71). No significant correlation between the dose of chloramphenicol succinate and serum concentrations of "free" chloramphenicol or the average urinary concentration were found. However, the average urinary concentration and serum concentration of oral chloramphenicol palmitate correlates well with dose indicating more complete and predictable hydrolysis. Variable fractions of the dose (a mean of 36%) was excreted in urine unchanged and was therefore not bioavailable in active form. Both variable hydrolysis and renal elimination of the nonhydrolyzed chloramphenicol succinate seems to reduce the bioavailability of the antibiotic and appears to contribute substantially to the wide variations in serum concentrations produced following an IV dose.

In premature infants, an increased bioavailability of chloramphenicol was a result of decreased rate of clearance of the succinate salt causing a greater fraction of the salt dose to be hydrolysed to chloramphenicol (106). With the data currently available, chloramphenicol is not advised during the breast feeding period. Chloramphenicol is excreted into breast milk, in some instances in concentrations which are 50% of blood levels (107). Single 1 gm oral doses produce peak milk levels at 3 hr, which are undetectable at 6 hr (108). Single dose of 500 mg orally given every 6 hr produced serum levels of 0.98 - 3.5 µg/ml in breast milk (109). Most infants do not have developed hepatic conjugation system for glucuronidation which could result in toxicity. Although chloramphenicol milk levels are not sufficient to induce the grey-baby syndrome, toxicity to the bone marrow may occur (110). Toxic effects in infants had been reported (111) during the breast feeding period.

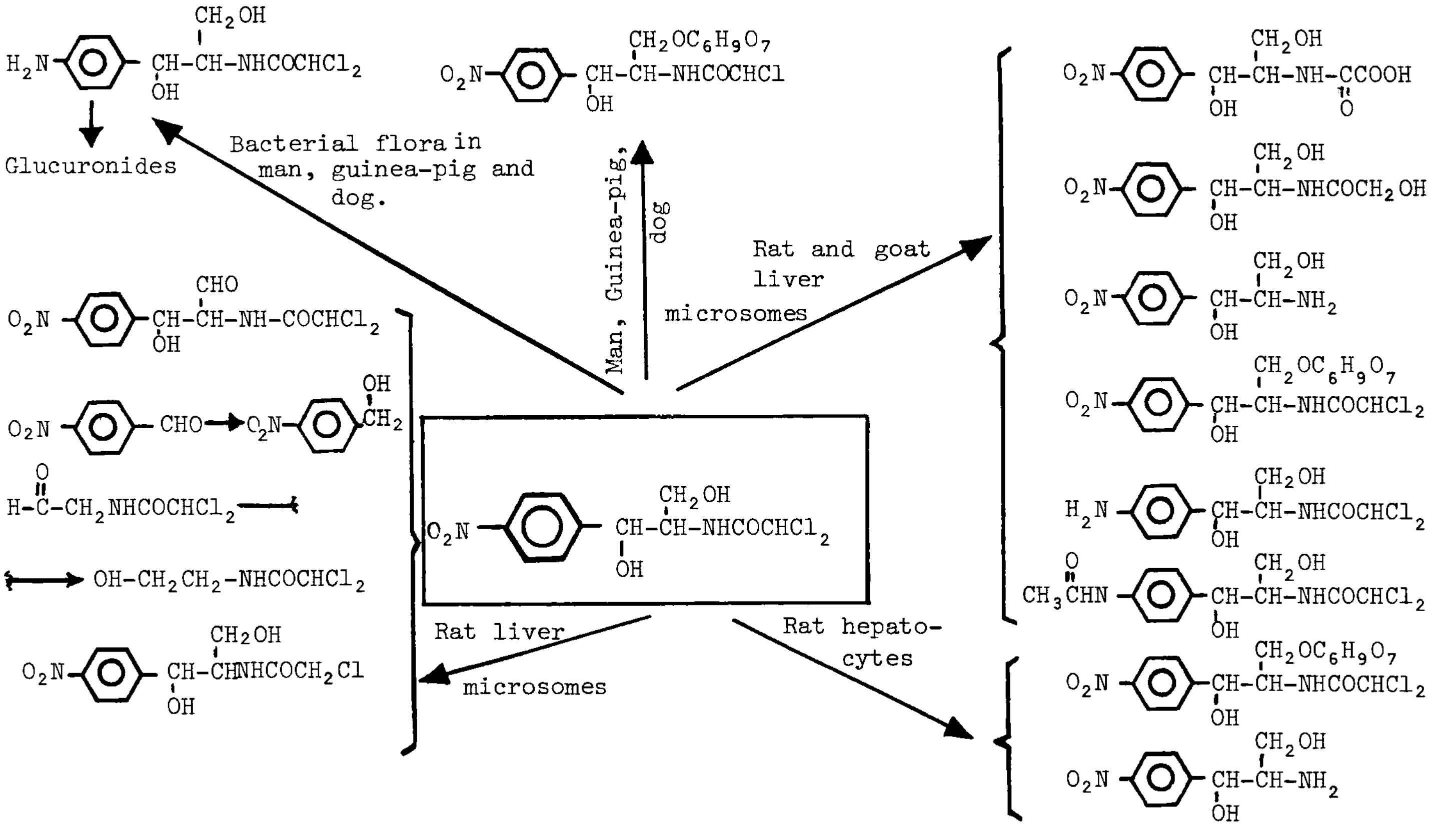
5.3 Half-Life

In normal, otherwise healthy adults, the half-life of chloramphenicol ranges from 1.6 - 3.3 hr with an

average value of 2.7 hr (102,112). In renal and liver failures the half-life is appreciably prolonged. In infants and children between the age of 1 month and 11 years, a mean apparent half-life was reported (113) to be 5.94 hr. Analysis of variance revealed a sample variance of 21.85 in children less than 4 months of age compared to 5.87 in children greater than 4 months of age reflecting a highly variable half-life in the younger infants. A study (114) of the pharmacokinetic parameters of chloramphenicol succinate in infants and young children revealed that half-life of serum chloramphenicol succinate did not correlate with the half-life of serum chloramphenicol. Chloramphenicol succinate is lost into the urine in significant quantity and this urinary loss must be taken into account in the estimation of chloramphenicol pharmacokinetic parameters. One duration of infusion of chloramphenicol succinate does not affect the amount excreted in the urine.

5.4 Metabolism

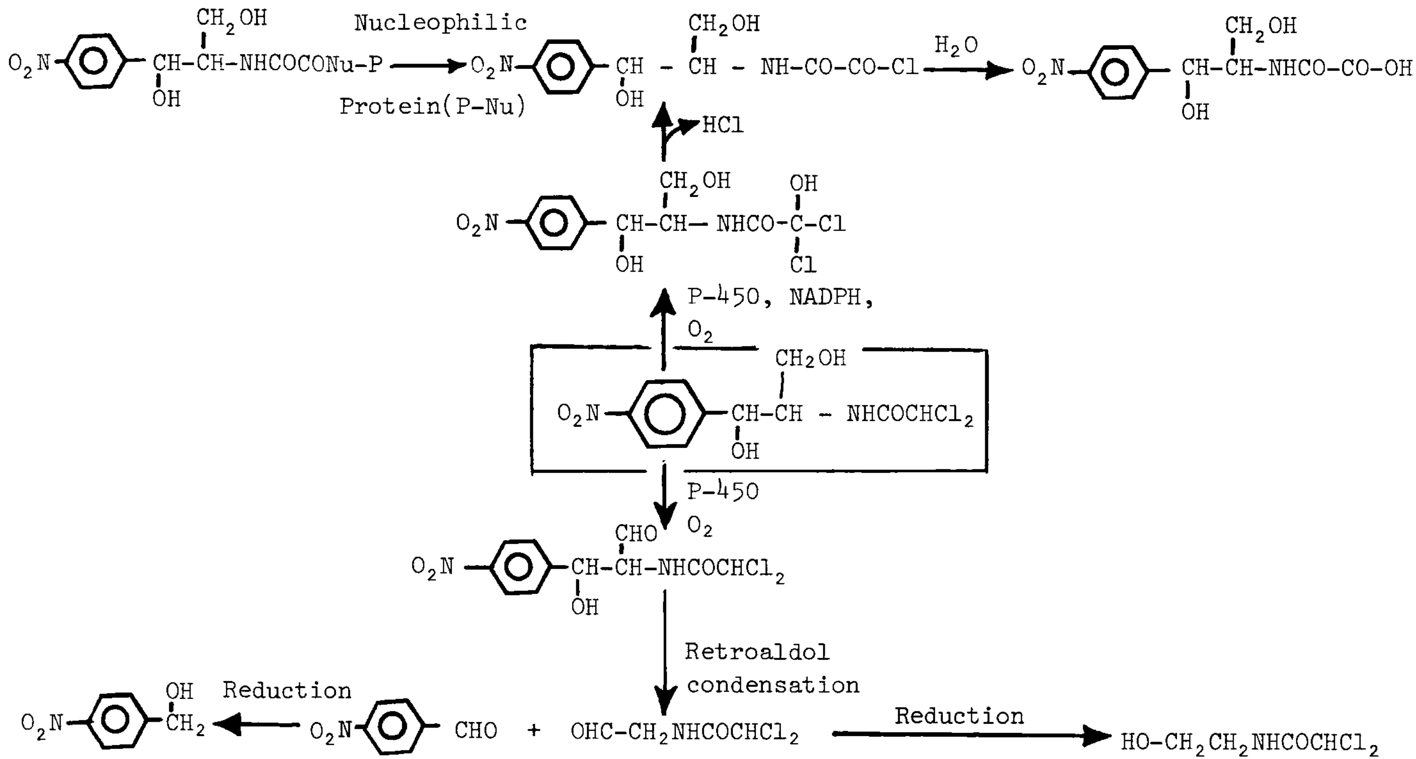
In early studies by Glazko *et al* (73,115,116) data on the fate of chloramphenicol in different species were produced. A major metabolic route involving glucuroconjugation as well as the reduction of the nitro group by the intestinal flora and conjugation of the resulting amines were described in rats, guinea pig and dog. In man, 90% of a single dose of of the drug appears in the urine within 24 hr, chiefly as chloramphenicol-3-glucuronide (117). The liver is the main site of glucuronidation. With the development of more sensitive analytical tools other metabolites of chloramphenicol have been suggested (Scheme 4). In their study of chloramphenicol metabolism in isolated rat hepatocytes Siliciano *et al* (118) chloramphenicol-3-glucuronide was found to be the major metabolite together with a minor metabolite believed to be D(-) *threo*-2-amino-1-(p-nitrophenyl)-1,3-propanediol. The formation of the 3-glucuronide was linear with respect to both the cell concentration and to the time of the first hour of incubation. The K_m and V_{max} values for the glucuronidation of chloramphenicol were 6.4×10^{-6} M and 420 pmol/min/10^8 cells respectively. The kinetics of the glucuronidation reaction in rat hepatocytes suggest a low hepatic extraction ratio of chloramphenicol.



Scheme 4. Metabolites of chloramphenicol in _in vivo_ and _in vitro_ studies.

The use of chloramphenicol has been shown to cause bone marrow depression. This toxicity is usually reversible if the drug is discontinued, but in rare cases (1 in 20,000) patients develop aplastic anemia a bone marrow disease which is often irreversible and fatal (119). Many mechanisms have been suggested (120-126) to account for this chloramphenicol-induced toxicity, but have not been unequivocally established. This rare incidence of chloramphenicol toxicity suggested to Pohl and Krishna (127) that a minor active metabolite may be involved in its induction. Using a cytochrome P-450 enzyme system in liver microsomes of rats, they studied the mechanism of the metabolic activation of chloramphenicol by measuring the covalent binding to microsomal proteins of specifically labelled [^{14}C] and [^{3}H] derivatives of chloramphenicol. The lack of binding of dichloroacetic acid, chloramphenicol base (2-amino-1-(p-nitrophenyl)-1,3-propanediol), and the acetamido and trifluoroacetamido derivatives of chloramphenicol indicates that the dichloroacetamido group is required for activation. The binding of dichloroacetamide support this conclusion. Moreover, the C-H bond of the dichloromethyl carbon of chloramphenicol appears to be broken in the activation process since the hydrogen is lost in covalent binding. Accordingly, a mechanism (Scheme 5) is proposed in which chloramphenicol is activated by hydroxylation of the dichloracetamido group followed by spontaneous dehydrochlorination to an oxamyl chloride which acylates microsomal proteins. Recently, it has been shown that cytochrome P-450 is the predominant protein is liver microsomes that is acylated by the oxamyl chloride (128). Moreover, the covalently modified cytochrome P-450 appears to be irreversibly inactivated as a mixed-function oxidase (129,130).

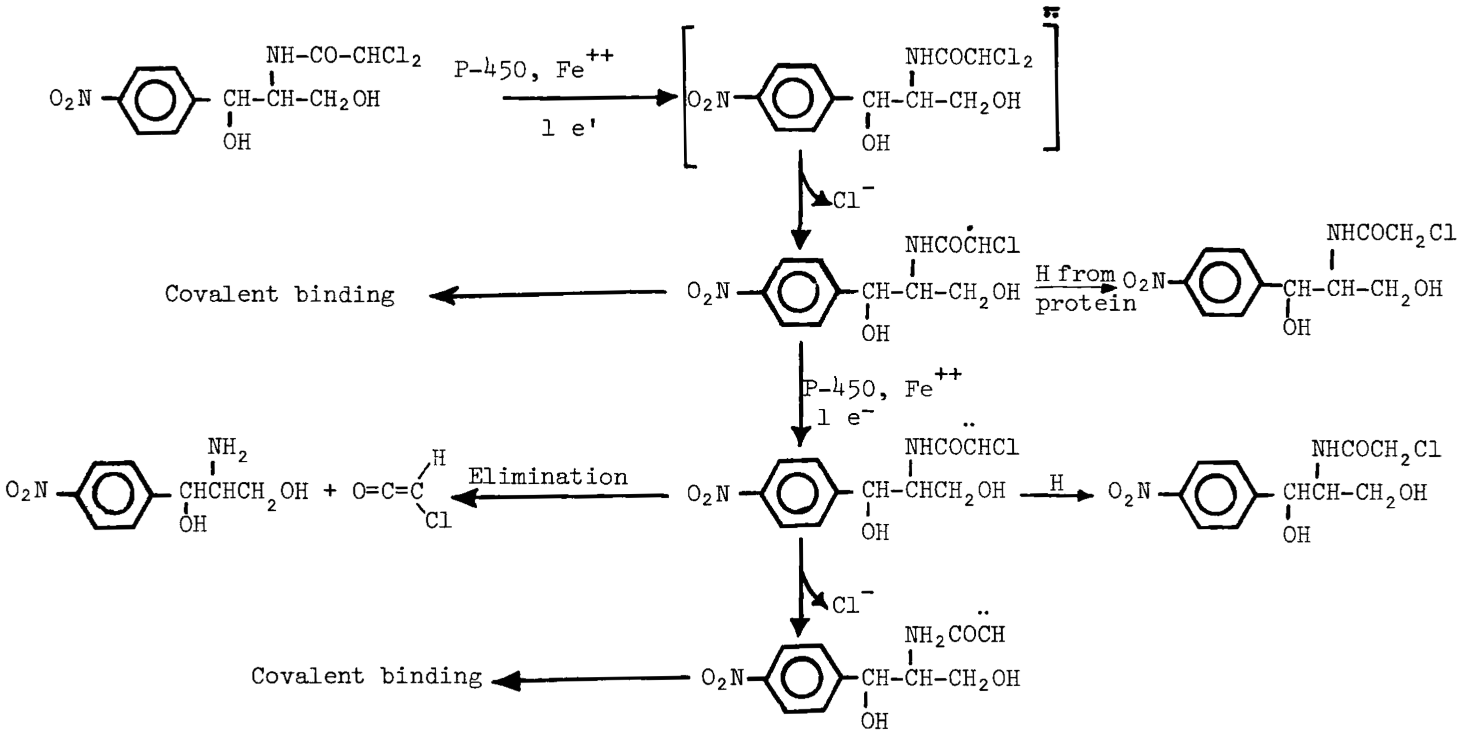
Morris et al (131) have further characterized the oxidative metabolism of chloramphenicol and have found a new pathway for the oxidative metabolism of chloramphenicol in addition to the oxidative dehalogenation reaction outlined above. According to these authors, when chloramphenicol was incubated with rat liver microsomes, four previously unidentified metabolites were detected and identified. They include chloramphenicol aldehyde, p-nitrobenzyl alcohol, N-(2-oxoethyl) dichloroacetamide, and N-(2-hydroxyethyl)



Scheme 5. Mechanism of chloramphenicol oxidative pathways by rat liver microsomes (127, 131).

dichloroacetamide. The formation of these metabolites was dependent upon the presence of NADPH and O_2 and was uninhibited when SKF 525A or CO/O_2 (8 : 2, v/v) were present in the reaction mixture. Moreover, the metabolites were formed by liver microsomes from phenobarbital-treated rats but not by microsomes from untreated rats or rats treated with β-naphthoflavone. The formation of these metabolites is consistent with a mechanism that involves an initial oxidation of chloramphenicol to chloramphenicol aldehyde by cytochrome P-450. The metabolite, being a β-hydroxyaldehyde, can chemically undergo a retro-aldol cleavage to p-nitrobenzaldehyde and N(2-oxoethyl)dichloroacetamide. Enzymatic reduction of these aldehyde intermediates would yield p-nitrobenzyl alcohol and N-(2-hydroxyethyl) dichloroacetamide, respectively (Scheme 5). In the above study by Pohl and Krishna (127) it was observed that only a 58% decrease in covalent binding of chloramphenicol metabolites to microsomal protein occurred when reactions were conducted in an atmosphere of nitrogen. At the time, it was felt that the reason the covalent binding was not decreased to an even greater extent was because of insufficient deoxygenation of the incubation mixtures. In a further study by Morris et al (132), however, it is shown that at low oxygen tension, chloramphenicol is activated by reductive dechlorination pathways of metabolism. Thus, when chloramphenicol was incubated with rat liver microsomes anaerobically, it was metabolized predominently to deschloro-chloramphenicol and products that become irreversibly bound to microsomal protein. Cytochrome P-450 induced by phenobarbital appeared to catalyse these reactions most effectively. Glutathione increased the formation of deschloro-chloramphenicol by 13% and decreased the amount of irreversibly bound product by 18%. Only small amount of the nitroaromatic-reduced product, chloramphenicol amine, was detected by HPLC. The results are consistent with the drug being biotransformed and activated by cytochrome P-450 anaerobically through predominently reductive dechlorination. A proposed mechanism for the reductive dechlorination is shown in Scheme 6.

Bories et al (133) developed a simple and ion-pair reverse phase high performance liquid chromatographic separations combined with selective extraction in



Scheme 6. A proposed mechanism for the reductive dechlorination of chloramphenicol by rat liver microsomes (132).

order to achieve an improved analytical tool for chloramphenicol metabolic profiling. Quantitation was achieved by summation of radioactivity values for fractions belonging to the same peak. Metabolites of chloramphenicol were identified by electron-impact and chemical ionization mass spectrometry. The study lead to the confirmation of previously suggested chloramphenicol metabolites as well as the identification of new metabolites.

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LIDOCAINE AND LIDOCAINE HYDROCHLORIDE

Michael F. Powell

1. Introduction

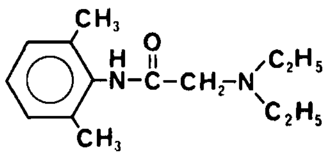
Lidocaine is widely used as a local anaesthetic and for the management of cardiac arrhythmas, particularly those associated with acute myocardial infarction.

The following supplement contains updated information pertaining to the analytical chemistry of lidocaine free base and lidocaine hydrochloride. A literature survey was conducted and is complete up to December 1985. The numbering system for topics discussed is the same as that in the original profile[1] (see Volume 14, pages 207-243).

2. Description

2.1 Nomenclature, Molecular Weights and CAS Numbers

The structural formula for lidocaine is given below.



A plethora of names for lidocaine have been used in the recent literature, for example: lignocaine, 2-(diethylamino)-N-(2,6-dimethylphenyl)-acetamide, 2-(diethylamino)-2',6'-acetoxyidide, N,N-diethyl-(2,6-xylylcarbamoyl)-methylamine and 2-diethylaminoaceto-2',6'-xylidide. The hydrochloride salt is named similarly but with the added suffix "hydrochloride" or "hydrochloride monohydrate". Trade names for lidocaine are: Lidocitin, Leostesin, Xylocaine, Xylotox, Xylestesin, Xylocitin, Rucaina, Duncaine, Isicaine and Anestacon.

Table 1. Description of Lidocaine and its Hydrochloride Salts

FORM	Free Base	Salt	Salt Monohydrate
Formula	$C_{14}H_{22}N_2O$	$C_{14}H_{22}N_2O \cdot HCl$	$C_{14}H_{22}N_2O \cdot HCl \cdot H_2O$
MW	234.34	270.80	288.81
%C,H,N	71.75,9.46,11.76	62.09,8.56,10.34	58.22,8.72,9.70
CAS Number	137-58-6	73-78-9	6108-05-0

3. Physical and Chemical Properties

3.2 Solubility

The solubility of lidocaine free base in aqueous solution is unusual in that it decreases as the temperature increases (Table 2).[2] This inverse temperature - solubility relationship has also been verified by others.[3-5] The pH dependence on the aqueous solubility of lidocaine can be calculated from the equation $S_T = S_{fb}(1 + 10^{(pK_a - pH)})$ where S_T and S_{fb} are the total and free base solubilities, respectively.[6] The calculated solubility of lidocaine at 14.9°, 25° and 37°C are shown in Figure 1. The solubility of lidocaine and lidocaine hydrochloride[7] in various solvents are given in Table 3.

Table 2. Free Base Solubility and pK_a Values of Lidocaine at Various Temperatures

Temperature (°C)	pK_a [8]	Solubility (mg/mL) [2]
10.0	8.24	
14.9		4.33 ± 0.12
25.0	7.92	3.81 + 0.02
34.5		3.42 ± 0.02
37.0		3.36 ± 0.02
38.0	7.57	

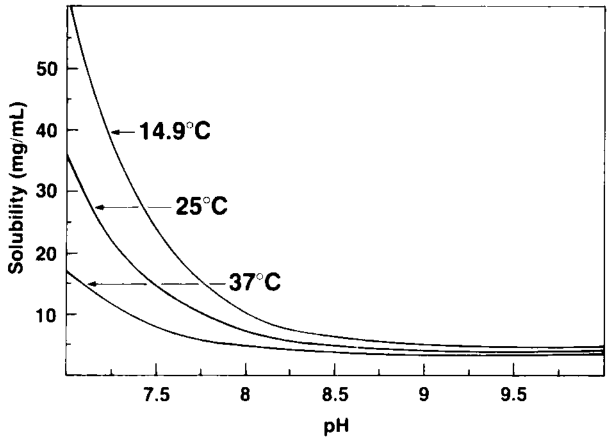


Figure 1. Calculated pH dependence of the aqueous solubility of lidocaine at 14.9°, 25° and 37°C. At pH 7.4, the calculated solubilities of lidocaine at 25° and 37°C are 16.4 and 8.7 mg/mL, respectively.

Table 3. Solubility of Lidocaine Free Base and Lidocaine Hydrochloride at 25°C in Various Solvents

Solvent	Solubility (mg/mL)	
	Lidocaine HCl	Free Base
Methanol	67	>500
Ethanol	11	>500
Acetone	1.8	>500
Chloroform	4	
Ether	<0.01	~500
Carbon tetrachloride	0.01	>500

3.3 Dissociation Constant

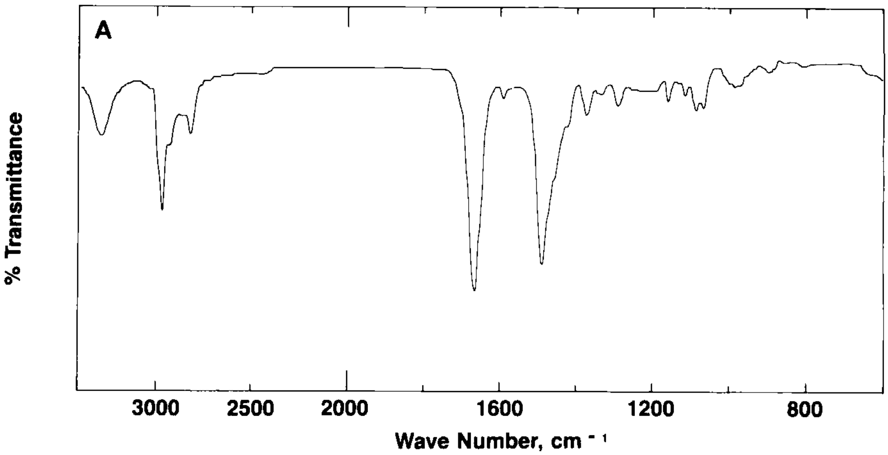
The pK_a of lidocaine has been measured by potentiometric titration at several temperatures at ionic strength 0.05 M (KCl),[8] and the data are shown in Table 2. The ionic strength, when adjusted with KCl from 0.005 to 0.077 M, showed little effect on the acidity constant.[9] The pH of a 5% (w/v) solution of lidocaine hydrochloride is approximately 4.0 to 5.5.

3.5 Infrared Absorption Spectra

The IR spectra of lidocaine and lidocaine hydrochloride were measured in chloroform using a Sargent Welsh Model S-200 IR spectrometer (Figure 2). Note that most of the stretching frequencies are shifted upwards than those obtained in KBr discs[1]. The structural assignments have been correlated with the following band assignments (Table 4).

Table 4. IR Band Frequencies (cm^{-1}) for Lidocaine and Lidocaine Hydrochloride Measured in $CDCl_3$.

Assignment	Lidocaine	Lidocaine Hydrochloride
N-H Stretch	3290	3180
C-H Stretch	2820	-
C-H Stretch	2970	2978
Amide I, C=O	1670	1682
Amide II, C-N	1495	1525
Fingerprint	812	949
region	900	978



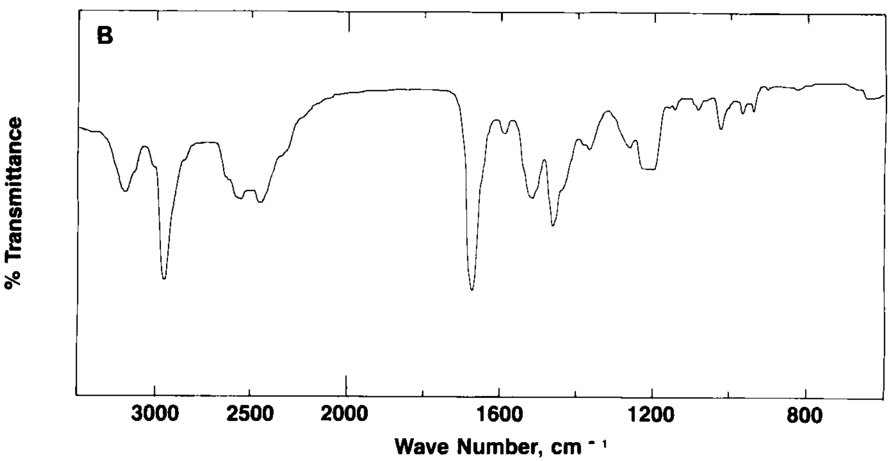


Figure 2. IR spectra of a) lidocaine and b) lidocaine hydrochloride in $CHCl_3$.

3.6 Nuclear Magnetic Resonance Spectra

The 90 MHz proton and 75.5 MHz ^{13}C-NMR spectra for lidocaine hydrochloride in $CDCl_3$ are shown in Figure 3. A summary of the proton chemical shifts as referenced to TMS are given in Table 5 and the ^{13}C-chemical shifts and relaxation times are given in Table 6. The numbering is as shown earlier in the structural formula. The ^{14}N-NMQR spectrum has also been reported.[10]

Table 5. Summary of Proton Chemical Shifts and Assignments for Lidocaine Hydrochloride in $CDCl_3$[a]

Assignment	Chemical Shift (ppm)
C_3-$\underline{H}$	7.02 (m)
C_4-$\underline{H}$	7.02 (m)
Ar-C$\underline{H}_3$	2.21 (s)
N-$\underline{H}$	10.24 (s-broad)
N-C(O)-C$\underline{H}_2$-N	4.22 (s)
N-C$\underline{H}_2$-CH_3	3.60 (q) J=7.20 Hz
N-CH_2-C$\underline{H}_3$	1.42 (t) J=7.20 Hz

[a]s = singlet, t = triplet, q = quartet, m = multiplet

Table 6. Summary of ^{13}C-NMR Data for Lidocaine Hydrochloride

Assignment	Relaxation Time[a] (s)	Chemical Shift (ppm)
N-$\underline{C}$(O)-CH_2-N	11.8	162.9
C_2, C_6	20.1	135.1
C_1	20.1	133.1
C_3, C_5	1.4	128.1
C_4	1.0	127.5
N-C(O)-$\underline{C}H_2$-N	1.0	51.0
N-$\underline{C}H_2CH_3$	1.2	49.1
Ar-$\underline{C}H_3$	3.1	18.7
N-$CH_2\underline{C}H_3$	2.0	10.0

[a]Lidocaine in $CDCl_3$ at 32°C in undegassed solution, Reference 11.

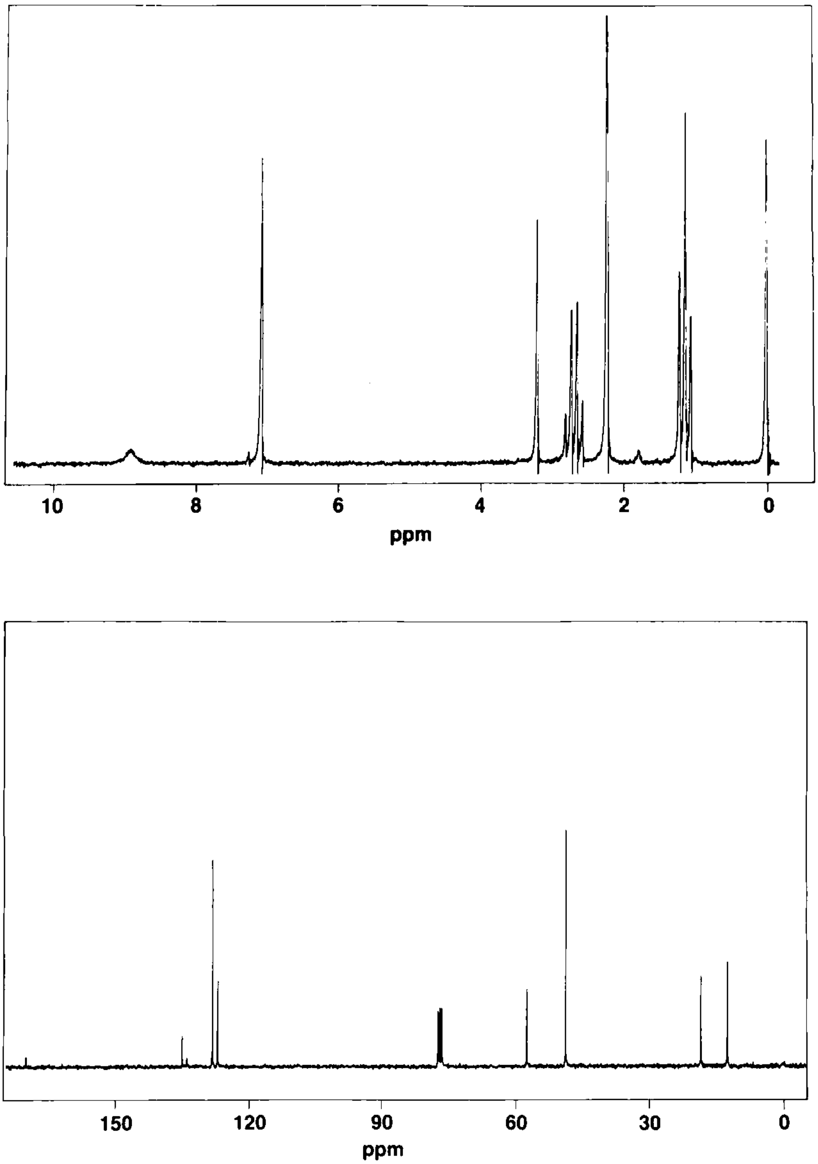


Figure 3. Proton NMR and ^{13}C-NMR spectra of lidocaine hydrochloride in $CDCl_3$.

3.9 X-Ray Crystal Structure

The crystal structure of lidocaine free base was determined from a single crystal grown from dimethylformamide - water solution at -20°C.[12] Cell dimensions were a = 13.24 Å, b = 14.06 Å, c = 19.25 Å and ß = 123.7° at 20°C. Both of the independent molecular conformations observed were in the trans-amide configuration.

X-ray crystal analysis of lidocaine hydrochloride was determined from a single crystal grown in acetone-ethylacetate.[13] The translucent prisms were of high mosaicity and gave the following cell parameters: a = 8.49 Å, b = 7.11 Å, c = 27.58 Å and ß = 106.87°.

3.10 X-Ray Diffraction

The X-ray powder diffraction patterns of lidocaine and lidocaine hydrochloride are shown in Figures 4a and 4b, respectively, and a summary of the scanning angles and relative intensities are given in Table 7. The data were collected using a Nicolet X-ray diffractometer equipped with a fine focus X-ray tube and a diffracting beam monochromator. The scanning angle was from 3° to 30° 2θ at 0.05°/second.

Table 7. X-Ray Powder Diffraction Data - Scanning Angles and Relative Intensities for Lidocaine and Lidocaine Hydrochloride

Lidocaine		Lidocaine Hydrochloride	
Degrees 2θ	Relative Intensity	Degrees 2θ	Relative Intensity
10.5	100	20.2	100
12.7	100	13.5	97
12.5	46	16.5	92
10.0	37	25.4	66
8.0	13	27.1	55
14.3	12	6.7	49
15.1	12	14.3	44

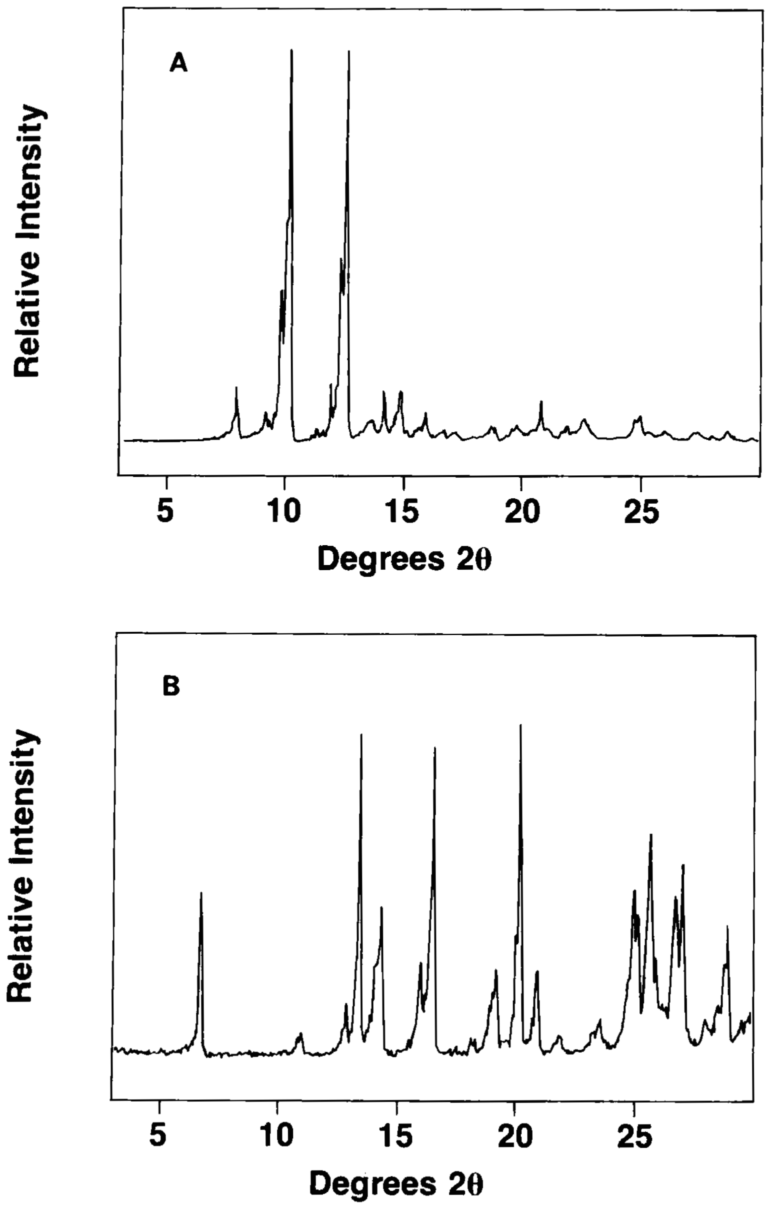


Figure 4. X-ray diffraction pattern of a) lidocaine and b) lidocaine hydrochloride.

3.11 Hygroscopicity

Lidocaine free base is not hygroscopic. For example, lidocaine free base does not absorb water even at 92% RH at room temperature. The anhydrous HCl salt however, does adsorb 1 mole of water per mole of lidocaine below 80% relative humidity at 25°C. At 93% relative humidity, more than two moles are adsorbed.[14] Limited hygroscopicity data for lidocaine hydrochloride from 22% to 90% relative humidity has also been reported.[15]

3.12 Self Association

Lidocaine in solution depresses only slightly the surface tension of water and thus, does not form micelles to any measurable extent.[16] However, lidocaine has been reported to form charge transfer complexes, for example with trinitrobenzene or chloropromazine.[17]

A controversy regarding inter versus intramolecular hydrogen bonding in lidocaine emerged in the early 70's and appears now to be resolved in favor of the latter. An earlier publication[18] postulated the acylic cis-amide configuration based on IR studies, however, subsequent IR studies[19] and NMR results[20] rebutted with strong evidence for intramolecular trans-amide hydrogen bonding as shown in Figure 5.

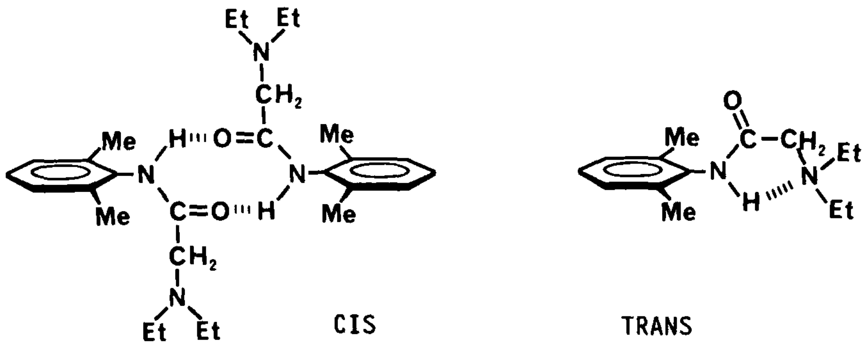


Figure 5. Cis- and trans-amide configurations of lidocaine.

6. Stability

6.2 Physical Stability in Parenteral and IV Solutions

Some lidocaine preparations are physically unstable resulting in solution cloudiness, precipitation of drug, or loss of drug potency in solution. Precipitation or cloudiness is usually caused by other additives which may complex with lidocaine or change the solution pH. Loss of drug potency in solution is not due to chemical instability, but is caused by adsorption of lidocaine to the container surface, especially when parenteral or IV solutions are stored in plastic containers (Table 8).

Table 8. Stability of Lidocaine in Parenteral Solutions

Solution	Container	Time (days)	Temp. (°C)	% Remaining	Reference
5% dextrose	plastic	120	4	99.0 ± 1.6	21
	plastic	120	30	100.2 ± 1.7	"
0.9% sodium chloride injection[a]	glass	14	25	100.9 ± 0.8	22
	plastic	14	25	101.9 ± 0.8	"
0.45% sodium chloride[b] and 5% dextrose[b]	glass	14	25	94.3 ± 0.3	"
	plastic	14	25	98.6 ± 0.9	"
Lactated ringers solution[a]	glass	14	25	101.9 ± 0.9	"
	plastic	14	25	101.9 ± 1.7	"
5% dextrose and lactated ringers solution[a]	glass	14	25	94.7 ± 0.8	"
	plastic	14	25	101.9 ± 0.9	"
Cardioplegic solution[b]	glass	21	22	90.1 ± 2.0	23
	plastic	21	22	25.6 ± 1.1	"
	glass	21	4	100.0 ± 2.2	"
	plastic	21	4	89.1 ± 5.8	"

[a]Admixture with aminophylline, bretylium tosylate, calcium gluconate, digoxin, dopamine hydrochloride, regular insulin, phenytoin sodium and procainamide hydrochloride.
[b]With lidocaine hydrochloride, KCl, $NaHCO_3$, dextrose and NaCl.

The first comprehensive paper on the compatibility of lidocaine in glass and plastic containers containing 5% dextrose in saline, normal saline, or lactated ringers solution reported a slight loss of lidocaine concentration after only twenty-four hours; this decrease was attributed to experimental error.[24] Subsequent reports showed that lidocaine is stable in parenteral solutions[21] providing certain admixtures are not included[22].

It has also been demonstrated recently that adsorption to polyvinyl chloride bags may occur, especially at room temperature.[21]

6.3 Photochemical Stability

The weak UV-visible absorption of lidocaine at $\lambda = 262$ nm has only a small molar absorptivity of 470 M^{-1} cm^{-1} and so lidocaine is not photoreactive. In our laboratory, Rayonet irradiation of a 5×10^{-5} M aqueous solution of lidocaine for 10 days resulted in only 5% loss of drug. Since the Rayonet reactor (Rayonet Model No. RPR-100) has been shown to accelerate most reactions by a factor of 100-200 times over indirect window light, the calculated shelf-life of lidocaine exposed to indirect window light is approximately eight years.

7. Pharmacokinetics

7.1 Plasma Concentrations after Different Routes of Administration

Lidocaine exhibits rapid and relatively high absorption after oral administration. However, it is not clinically useful when taken orally due to extensive first-pass metabolism and its short biological half-life. It was reported that after oral administration of lidocaine to rats, dogs and man, only 0.2%, 2.0% and 2.8%, respectively, was recovered unchanged in the urine[25] (Table 9). The mean apparent oral absorption of lidocaine in normal subjects was approximately 35%,[26] in close agreement with the reported hepatic extraction ratio.[27] This is contrary to an earlier report in which the colorimetric method used for analysis may not have been specific for unmetabolized lidocaine.[28] A prediction of the bioavailability using oral clearance data has also been offered.[29] Plasma levels obtained from ingesting 500 mg of lidocaine appeared to be less than the levels required to cause an antiarrhythmic effect.

Table 9. Tissue Distribution After Lidocaine p.o. Dosing of 10 mg/kg to Rats.

Tissue	Total Radioactivity			Unchanged Lidocaine[a]		
	0.5 h	2 h	24 h	0.5 h	2 h	24 h
Stomach	13.7	3.3	0.0	14.1	2.7	0.0
Intestine	27.9	45.3	6.9	2.4	0.3	0.0
Blood	2.8	2.1	1.6	0.2	0.0	0.0
Feces	0.0	0.2	0.5	n.d.	n.d.	n.d.
Brain	0.6	0.3	0.1	0.0	0.0	0.0
Liver	11.7	3.4	1.1	0.3	0.0	0.0
Spleen	0.4	0.0	0.0	0.1	0.0	0.0
Kidney	2.0	0.8	0.2	1.3	0.1	0.0
Heart	0.2	0.1	0.5	0.0	0.0	0.0
Lung	1.1	0.3	0.3	0.2	0.0	0.0
Carcass	40.4	24.2	20.8	b	b	b
Urine	6.1	24.6	73.0	0.3	0.5	0.3

[a]Unchanged lidocaine as calculated for the hydrochloride salt. [b]not determined.

Intravenous administration of lidocaine to rats results in rapid uptake by the highly perfuse organs such as the liver (which is considered the primary metabolic site), heart, lungs, brain and kidneys (Table 9).[25] The elimination half-life of lidocaine in rats is 30 minutes and in dogs is 45-60 minutes.

After IV injection, the mean half-life in plasma of normal subjects is approximately 6 minutes and the elimination half-life is ~100 minutes.[30] A rapid IV injection of 160 mg lidocaine into normal subjects followed by a 4 mg/min infusion resulted in a mean initial plasma level of 2.6 μg/mL which decreased to a steady state plasma level of 2-4 μg/mL after approximately 20 minutes. It has been shown using averaged data from several patients that an initial bolus of 125 mg and an infusion of 0.8 mg/mL maintained plasma levels of 1 μg/mL in a 70 kg man. When infusion was given alone, plasma levels of lidocaine increased until a plateau was reached, i.e. when input equaled output. This usually took three to four half-lives or from five to seven hours. Effective concentrations of lidocaine for local anaesthesia were achieved for approximately one hour by a single 200 mg intramuscular injection.[31]

Lidocaine proved to be slightly more bioavailable when administered rectally rather than orally.[32] This drug is also absorbed well through intact mucous membranes, skin and damaged tissue.[33] The skin permeability was greatly enhanced in in-vitro experiments by the addition of N,N-diethyl-m-toluamide.[34]

A review of the clinical pharmacokinetics of lidocaine has been published recently,[35] and a synopsis of effects with respect to pregnancy and the neonate appears regularly in Martindale, The Extra Pharmacopoeia.[7]

7.3 Biotransformation and Elimination

Two of the first-formed metabolites, ethylglycinexylidide and glycinexylidide, showed 83% and 10% of the antiarrhythmic activity of lidocaine, respectively.[36] The aryl-hydroxylated analogues of these compounds were also formed and were usually found as acid-hydrolyzable 'conjugates'.[25] It was originally proposed[37] that N-deethylation preceded hydroxylation but recent studies in rats have shown that i) both N,N-diethyl and N-monoethyl glycine are formed, ii) lidocaine and monoethylglycin exylidide show competitive inhibition[38] and, iii) the 3-hydroxylation and N-deethylation are catalyzed by different P_{450}-dependent enzymes.[39,40] The metabolic product distributions in rats, guinea pigs, dogs, and man are shown in Table 10.

Table 10. Summary of Metabolic Products of Lidocaine

Compound	% Dose Recovered in 24 h Urine[a]			
	Rat	G. Pig	Dog	Man
Lidocaine	0.2	0.5	2.0	2.8
Ethylglycinexylidide	0.7	14.9	2.3	3.7
Glycinexylidide	2.1	3.3	12.6	2.3
3-Hydroxy-lidocaine	31.2	0.5	6.7	1.1
3-Hydroxy-ethylglycinexylidide	39.6	2.0	3.1	0.3
2,6-Xylidine	1.5	16.2	1.6	1.0
4-Hydroxy-2,6-xylidine	12.4	16.4	35.2	72.6
Total	85.0	53.8	65.5	83.8

[a]P.O. doses given to rat, guinea pig, dog and man were 20, 20, 10 and 3 mg/kg, respectively. Because of the relatively large differences in molecular weights of the lidocaine metabolites, doses and recoveries were determined as molar concentrations of the free bases.

8. Methods of Analysis

8.1 Reverse Phase HPLC

Although the analysis of ionic and strongly basic drugs by RP-HPLC is not without problems such as long solute retention or peak-tailing, it was estimated some time ago that approximately eighty percent of all liquid chromatography analyses are carried out using reverse phase methods.[41] The four examples offered here were chosen because of their ability to separate lidocaine from its degradation products or common pharmaceuticals under a variety of conditions.

System I[42]

Column:	30 cm x 4 mm ID µ-Bondapak-CN, 10 µm
Detection:	Dual channel, fixed wavelength, Waters Model 440
Temperature:	Ambient
Flow Rate:	2 mL/min
Mobile Phase:	0.01 M Octanesulfonic acid sodium salt, 0.01 M edetate disodium, 2% (v/v) acetic acid, 2% acetonitrile and 1% methanol in distilled water
Retention Time:	7 min
Separation:	From degradation products and epinephrine

System II[43]

Column:	Nucleosil 100, 10µm
Detection:	Fixed wavelength, 254 nm
Temperature:	25.0 ± 0.1°C
Mobile Phase:	Sodium phosphate buffer, pH 2.2, µ = 0.1 and 1% (v/v) 1-pentanol
Retention Time:	7 min
Separation:	From other local anaesthetics

System III[44]

Column:	Li Chromsorb RP-18
Detection:	Fixed wavelength, 254 nm
Temperature:	25°C
Flow Rate:	Not given
Mobile Phase:	0.01 M Tetramethylammonium bromide, 20% water, 80% methanol, pH 3.6 with H_3PO_4
Separation:	From pharmaceuticals having basic functional groups

System IV[45]

Columns:	μ-Bondapak C_{18}, μ-Bondapak Phenyl, μ-Bondapak-CN, μ-Bondagel, Chromegabond C_8 and Chromegabond C_6H_{11}
Detection:	UV-Vis detector
Flow Rate:	1.5 mL/min
Mobile Phase:	Methanol:water:acetic acid (29:50:1) containing .005 M heptane sulfonic acid sodium salt, pH ~ 4
Retention Time:	Retention volumes for the various columns given in Reference 45
Separation:	From other common pharmaceuticals

8.2 Normal Phase HPLC

System I[46]

Columns:	125 or 250 x 4.9 mm ID column packed with Spherisorb 55 W silica, Syloid 74 silica
Detection:	215 nm
Temperature:	Ambient
Flow Rate:	2 mL/min
Mobile Phase:	Methanol containing 1.85 mM $HClO_4$
Retention Time:	7 min
Separation:	From other local anaesthetics

8.3 Thin Layer Chromatography (TLC)

TLC and high performance TLC are often used for drug analysis because they are rapid, inexpensive, and only require small amounts of sample. Lidocaine is identified on the TLC plate by either short wavelength UV light or by a positive test with acidified iodoplatinate spray. For example, the following systems have been used:

System I[47]

Plate:	Silica gel GF-254, 0.25 mm
Temperature:	Ambient
Detection:	UV
Mobile Phase:	$CHCl_3$/ether/MeOH/conc. NH_4OH (15:25:5:1)
R_f:	0.80

System II[47]

Plate:	Silica gel GF-254, 0.25 mm
Temperature:	Ambient
Detection:	UV
Mobile Phase	Ethyl acetate/n-propanol/conc. NH_4OH (40:30:3)
R_f:	0.82

System III[47]

Plate:	Silica gel GF-254, 0.25mm
Temperature:	Ambient
Detection:	UV
Mobile Phase:	MeOH/conc. NH_4OH (100:1.5)
R_f:	0.84

System IV[47]

Plate:	Silica gel GF-254, 0.25mm
Temperature:	Ambient
Detection:	UV
Mobile Phase:	Alcohol USP/acetic acid/water (60:30:10)
R_f:	0.60

System V[48]

Plate:	Silica gel G60 F_{254} (untreated)
Temperature:	Ambient
Detection:	UV
Mobile Phase:	0.01 M KBr in methanol
R_f:	0.84 (0.54, plate treated with 0.1 M $KHSO_4$)

Acknowledgements

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SODIUM NITROPRUSSIDE

Auke Bult, Oscar R. Leeuwenkamp and Wouter P. van Bennekom

1. HISTORY

Nitroprusside was first described in 1849 by Playfair (1). The compound has attracted considerable interest at various periods in chemical history. At present, there are still controversies on various points of its physical and (photo)chemical properties.

The pharmaceutical interest in nitroprusside is due to its applications as analytical reagent and as strong hypotensive agent. Boedeker (2) introduced in 1861 nitroprusside as reagent for the detection of sulphite. Later on, Legal (3) extended its use as reagent to the detection of ketone bodies in urine of diabetic patients. Simon (4) and Rimini (5) employed the substance for detection of secondary and primary aliphatic amines, respectively.

The blood-pressure lowering effect of nitroprusside was already reported in 1887 by Davidsohn (6), while the first clinical trial was described in 1928 by Johnson (7).

This profile is supplementary to the profile of Rucki (literature was surveyed through October 1976) (8).

2. DESCRIPTION

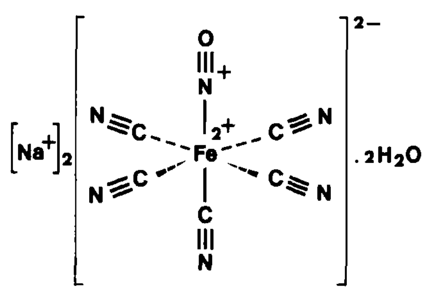
2.1 Name, Formula, Molecular Weight

Generic name - Sodium nitroprusside

Nomenclature - The following nomenclature is used in Chemical Abstracts: Ferrate(2-), pentakis(cyano-C)nitrosyl, disodium (OC-6-22)-[14402-89-2]

Synonyms - Sodium nitroferricyanide, sodium nitroprussiate, disodium pentacyanonitrosylferrate(II) dihydrate

Trade Names - Nipride® (Roche), Nipruss® (Cedona)



$C_5N_6OFeNa_2 \cdot 2H_2O$ Molecular Weight: 297.95

3. PHYSICAL PROPERTIES

3.1 ^{13}C-NMR Spectrum

The ^{13}C-NMR spectrum of nitroprusside in D_2O is simple. The equatorial and axial CN appear with chemical shifts of 104.2 and 102.2 ppm referred to t-butanol, respectively (9,10). These shifts are about 40 ppm lower as compared to $Fe(CN)_5X$ compounds ($X = NH_3$, H_2O, CN^-) and indicates a strong $d\pi$-backdonation to the NO^+ ligand (10).

3.2 Mössbauer Spectrum

Data on the Mössbauer spectrum of nitroprusside were presented by Danon (11). The isomer shift (σ) and quadrupole coupling (ΔE) have values of -0.012 cm s^{-1} and 0.185 cm s^{-1}, respectively.

3.3 Molecular Orbital Diagram

The MO diagram of nitroprusside is important for the interpretation of its physical and photochemical properties. Fig. 1 presents the partial MO-energy level diagram as calculated with the SCCC-MO method (12). All d-electrons are paired and the compound is therefore diamagnetic. The distribution of the electrons implies that the nitrosyl group has a formal charge +1 and iron +2 (d^6). The positive charge of NO explains the relatively high value of $\nu(NO) = 1940$ cm^{-1} and the reactivity of this group towards a wide variety of nucleophilic agents as well (13). The MO diagram is also illustrative for the assignment of the UV-VIS spectrum as presented in Table 1 (12).

Recently, a re-interpretation of the MO diagram based on Scaled INDO calculations has been published (14). Now bands I to III transitions are considered to be due to d-d and internal transitions in the ligands, while band IV is an allowed charge-transfer band.

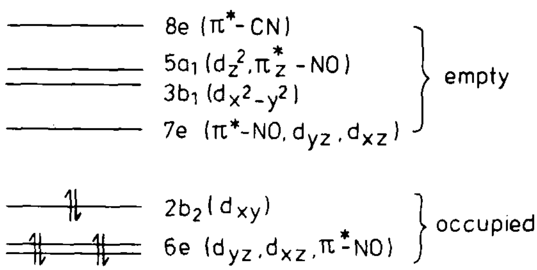


Fig. 1. Energy level diagram of the nitroprusside ion (12).

Table 1. Assignment of the UV-VIS spectrum

band	λ_{max}(nm)	ϵ(M^{-1} cm^{-1})	assignment
I	498	8	$2b_2 \rightarrow 7e$
II	396	25	$6e \rightarrow 7e$
III	330	40	$2b_2 \rightarrow 3b_1$
IV	265	900	$6e \rightarrow 5a_1$
V	238	700	$6e \rightarrow 3b_1$

A remarkable phenomenon of nitroprusside is that excitation with a suitable laser at T < 150 K results in the formation of an extremely long-living metastable state (15).

4. STABILITY AND DEGRADATION

Two important pathways of photochemical degradation of nitroprusside are (8):

$$[Fe(CN)_5NO]^{2-} + H_2O \xrightarrow{h\nu} [Fe^{II}(CN)_5H_2O]^{3-} + NO^+ \qquad [A]$$

and

$$[Fe(CN)_5NO]^{2-} + H_2O \xrightarrow{h\nu} [Fe^{III}(CN)_5H_2O]^{2-} + NO \qquad [B]$$

Recently the mechanism of photodecomposition has been re-examined with continuous and flash photolysis (16,17). The conclusions are: 1) band I is photo-inactive, 2) band II irradiation results in pathway [A] and 3) bands IV and V irradiation gives pathway [B] (for bands see Table 1). Owing to the overlap of band III with bands II and IV, both pathways can occur simultaneously. The conclusions are consistent with the MO diagram of Golebiewski and Wasielewska (14).

For clinical use sodium nitroprusside has to be reconstituted with a sterilized aqueous solution resulting in a concentrated stock solution (ca. 25 g l^{-1} in 0.9% saline or 5% glucose), which is diluted to an infusion solution (ca. 50-200 mg l^{-1} in 5% glucose). Both solutions have been studied for thermal and photochemical stability.

Protected from light, the concentrated solution is stable at room temperature and 4°C for more than two years (18). Autoclaving (15 min, 121°C) of nitroprusside solutions in water and in 0.9% saline (50 mg l^{-1}) gives essentially no degradation, whereas sterilization of nitroprusside in 5% dextrose solution results in about 40% loss (19). Nitro-

prusside solutions in light-protected glass or plastic containers and in plastic infusion sets remain stable for at least two days (20).

In solution nitroprusside is highly light-sensitive and decomposes rapidly (8). Modern photodegradation studies are based on "stability-indicating" assay methods for nitroprusside, viz. colorimetric measurement of intact nitroprusside with sulphide (18) and HPLC (19-22). The rate of degradation upon exposure to daylight and to light of 350 nm is essentially the same in water, in 0.9% saline and in 5% dextrose solutions (19). The degradation is a non-linear process, i.e. an initial rapid loss is followed by a phase of slower decomposition (19). The non-linearity is due to the formation of $[Fe(CN)_5H_2O]^{2-}$, a yellow compound acting as a light-filter. In addition, the back-formation of nitroprusside from its degradation products nitrite and $[Fe(CN)_5H_2O]^{3-}$ occurs according to (19):

$$[Fe(CN)_5H_2O]^{3-} + NO_2^- + 2\ H^+ \longrightarrow [Fe(CN)_5NO]^{2-} + 2\ H_2O$$

The degradation yields nitrite, nitrate, $[Fe(CN)_5H_2O]^{2-}$, $[Fe(CN)_5H_2O]^{3-}$, $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ (19). Contrary to previous reports, the addition of citric acid or sodium edetate (both 0.25 mg/100 ml) to 5% dextrose solution of nitroprusside (50 mg l^{-1}) does not improve the stability, while cyanocobalamin (10 mg l^{-1}) significantly increases the photo-stability (19). Before the introduction of the stability-indicating assay of nitroprusside, the degradation studies were based on the spectrophotometric measurement of the increase in absorbance at 395 nm. However, many degradation products of nitroprusside exhibit high molar absorptivities in that region, resulting in contradictory interpretations of the results (23-26). Very recently a study on the photodegradation of nitroprusside was published in which the release of HCN and the formation of $Fe(CN)_6^{3-}$ were measured colorimetrically with detection limits of 10-12 μM and 2-3 μM, respectively (27). Dimethyl sulfoxide (10% v/v) was lately found to be an effective photoprotective agent for solutions of nitroprusside (28,29).

5. PHARMACOLOGY AND IN-VITRO DEGRADATION

5.1 Pharmacology

The main pharmacological and toxicological features of nitroprusside have been discussed before (8). Recent reports in this field are indicative for the continued interest in this compound (30-37).

5.2 In-Vitro Degradation

In-vitro studies have revealed that cyanide is released from nitroprusside on incubation with serum, plasma, whole blood, liver homogenate, haemoglobin and erythrocytes (30). For instance, nitroprusside incubated with blood releases 50% of the total amount of cyanide within 20 min and more than 90% over 2 h (38). Upon infusion, in-vivo cyanide levels up to 3.2 μM and 45.5 μM were found in plasma and blood of patients, respectively (26).

The number of methods suitable for measurement of low concentrations of intact nitroprusside and its degradation products (cyanide, nitric oxide/nitrite) in body fluids is very limited (26,39-41). Arnold et al. (26) described an indirect colorimetric method, based on a diazo coupling reaction, for measurement of nitric oxide released from nitroprusside.

The cyanide determination is based on colour formation with pyridine/pyrazolone (39). This method is suitable for the determination of free cyanide (released from nitroprusside) and intact nitroprusside via quantitative conversion to cyanide by incubation with cysteine. The detection limit amounts to about 3 μg ml^{-1} of nitroprusside (39).

Recently Alkayer et al. (40) and Leeuwenkamp et al. (41) developed polarographic methods for the direct determination of nitroprusside in biological matrices with detection limits of 450 ng ml^{-1} and 15 ng ml^{-1}, respectively (for details see Section 6.5). Especially the last-mentioned procedure enables measuring of therapeutic levels of nitroprusside, ranging from 100 to 1000 ng ml^{-1} (41).

Leeuwenkamp (42) employed the polarographic method in a study of the in-vitro degradation of nitroprusside (200 ng ml^{-1}) in various media at 37°C, viz. solutions of albumin, cysteine, glutathione and (met)haemoglobin; human plasma, erythrocyte suspensions and blood, and in 100,000 g crude aortic-soluble fractions. A half-life time ($t_{1/2}$) of about 2.6 min was found in the soluble fractions, which is comparable to the in-vivo $t_{1/2}$. The $t_{1/2}$ in human blood was about 15 min and in the other mentioned media considerably higher values of $t_{1/2}$ were observed.

6. METHODS OF ANALYSIS

6.1 Identification Tests

The identification tests for sodium nitroprusside and its dosage form "sterile sodium nitroprusside" are the same in USP XXI as in USP XIX (8). Recently a new precipitation reagent for the nitroprusside anion has been described (43).

6.2 Purity Tests

The purity tests in USP XXI for sodium nitroprusside comprise chloride and sulphate; both tests are based on turbidimetric measurements. The limit test for determination of the hexacyanoferrates $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ at 0.05% level can be performed by chromatographic separation of sodium nitroprusside from these substances, followed by spectrophotometric measurement at 415 nm (44). Moreover, the chromatographic procedure described by Leeuwenkamp et al. (21) enables on-line determination of both hexacyanoferrates.

6.3 Colorimetry

Determinations of nitroprusside, based on a quantitative release of nitric oxide and colorimetric measurement of the nitrite formed by a diazo coupling reaction have already been described in Section 5.2 (26). Similar procedures have recently been reported (45,46).

Nitroprusside determination, based on a quantitative release of cyanide and subsequent colorimetric measurement of cyanide has been described in Section 5.2 (39).

Burger (47) reported the determination of cyanoferrate compounds, e.g. nitroprusside, by complete degradation of these compounds in a mixture of phenanthroline/ascorbic acid/mercury(II) chloride/acetate buffer (pH = 3.5) and quantitation of the released HCN by titration with silver nitrate and colorimetric measurement of Fe^{2+} as ferroin at 510 nm.

6.4 High-Performance Liquid Chromatography

Up to now three HPLC procedures have been described in the literature (20-22). Baaske et al. (22) used a reversed-phase ion-pair system with a stationary phase of microparticulate 10 μm phenyl-bonded silica gel and a mobile phase consisting of acetonitrile/phosphate/tetra-n-butylammonium hydroxide buffer of pH = 7.1 (30+70); UV-detection at 210 nm; linear calibration curve in the concentration range: 10-50 μg ml^{-1}; coefficient of variation (CV) < 3.1%. The system separates nitroprusside from its degradation product $Fe(CN)_6^{4-}$, but nitroprusside and $Fe(CN)_6^{3-}$ are not separated.

A similar system was described by Leeuwenkamp et al. (21). They used μ-Bondapack phenyl-bonded pellicular silica gel (10 μm) as stationary phase and a mobile phase consisting of water-methanol (65+35) containing 5 mM tetra-n-butyl-ammonium phosphate, 1.1 mM n-octylamine and 6.5 mM potassium dihydrogen phosphate (pH = 7.0); UV-detection at 220 nm; linear calibration curve in the concentration range:

10-120 μg ml^{-1}. This system enables the separation of nitroprusside from its photodegradation products NO_2^-, NO_3^-, $Fe(CN)_6^{3-}$, $Fe(CN)_6^{4-}$, $[Fe(CN)_5H_2O]^{2-}$ and $[Fe(CN)_5H_2O]^{3-}$ (19).

Most recently an HPLC procedure has been published (20) in which Partisil 10-SAX anion-exchange material is used as stationary phase with a mobile phase consisting of 0.5 M potassium dihydrogen phosphate buffer (pH = 3.0); UV-detection at 230 nm; linear calibration curve in the concentration range 1-200 μg ml^{-1}; CV < 1%. However, with this system no degradation products were detected.

6.5 Polarography

The key features of the polarographic behaviour of nitroprusside have been discussed before (8). In the pH range 4-9 at the dropping mercury electrode, three reduction processes (I-III) were observed with differential pulse polarography (DPP) and high-performance differential pulse polarography (HPDPP) with peak potentials at -360 (I), -610 (II) and -1500 (III) mV vs. saturated sodium chloride calomel electrode (SSCE) (48). At pH > 10 the polarographic diffusion-current decreases most probably because of formation of $[Fe(CN)_5NO_2]^{4-}$ (49). At pH = 6 peak II starts to shift anodically and increases in intensity with a decrease in pH (48). Peak II superimposes peak I at pH < 1.5 and the resulting peak shifts anodically in a linear way down to pH = 0 (48). At pH = 0 (1 M $HClO_4$) the combined reduction peak appears at -270 mV vs. SSCE and maximum sensitivity is obtained (linear calibration curve in the concentration range 4-1000 ng ml^{-1}) (48).

An extensive analysis of the reduction processes is given elsewhere (23,48-51). Lately, the one-electron reduction product of nitroprusside, viz. $[Fe(CN)_5\dot{N}O]^{3-}$ (I), has attracted considerable attention because of the rapid equilibrium (50-53):

$$\underset{\mathrm{I}}{[Fe(CN)_5\dot{N}O]^{3-}} \rightleftharpoons \underset{\mathrm{II}}{[\dot{F}e(CN)_4NO]^{2-}} + CN^-$$

The reduction product I decomposes to a reactive pentacoordinate complex (II) due to release of cyanide, while the unpaired electron transfers from the nitrosyl N atom to the iron atom. Compound II forms the hexacoordinate complex $[Fe(CN)_3(chel)NO]^-$ with suitable bidentate ligands (chel), e.g. 2,2'-bipyridine, 1,10-phenanthroline (54).

Polarography is used to quantitate nitroprusside in dosage form (USP XXI) and in body fluids (40,41). Alkayer et

al. (40) developed a determination method for nitroprusside in human serum based on phase-sensitive sine-wave polarography at pH = 7.4 after protein elimination with perchloric acid (linear concentration range 2-24 μg ml^{-1}). Leeuwenkamp et al. (41) based their method on HPDPP and DPP at pH = 0 (1 M perchloric acid). The linear calibration range in plasma, serum and blood (protein elimination by perchloric acid) is 30-1000 ng ml^{-1}.

6.6 Application of Nitroprusside as Analytical Reagent

Nitroprusside is a valuable reagent for the detection and determination of a wide variety of nucleophilic agents, e.g. primary and secondary aliphatic amines, aldoximes, amino acids, anilines, indols, ketones, nitrils, phenols, pyrroles, quinones, sulphite, thiols, thioureas and uracils (13,23,55-58). Its reactivity is based on the positive nitrosyl group, reacting with nucleophilic species (mostly in alkaline medium) (13). Most addition compounds formed are highly coloured and sufficiently stable to be used as a basis for spectrophotometric determination. The reaction mechanisms are (sometimes) rather complicated (23).

6.7 Miscellaneous Methods of Analysis

Low concentrations of nitroprusside (detection limit ca. 3 ng ml^{-1}) have been assayed by virtue of its catalytic effect on the formation of indophenol blue (λ_{max} = 620 nm) from ammonia, phenol and hypochlorite in alkaline medium (reaction of Berthelot) (59).

Nitroprusside can be determined by flow-injection analysis with amperometric detection (oxidation at a glassy-carbon electrode or reduction at a sessile mercury drop electrode); concentration range 10^{-6} to $5 \cdot 10^{-3}$ M; CV < 2% (60).

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CUMULATIVE INDEX

Bold numerals refer to volume numbers

ERRATUM TO
K. Florey (Ed.). (1985). Analytical Profiles of Drug Substances, vol. 14.

Page 82, Table 1:
The molar extinction coefficient at 285 nm is 109, not 190.